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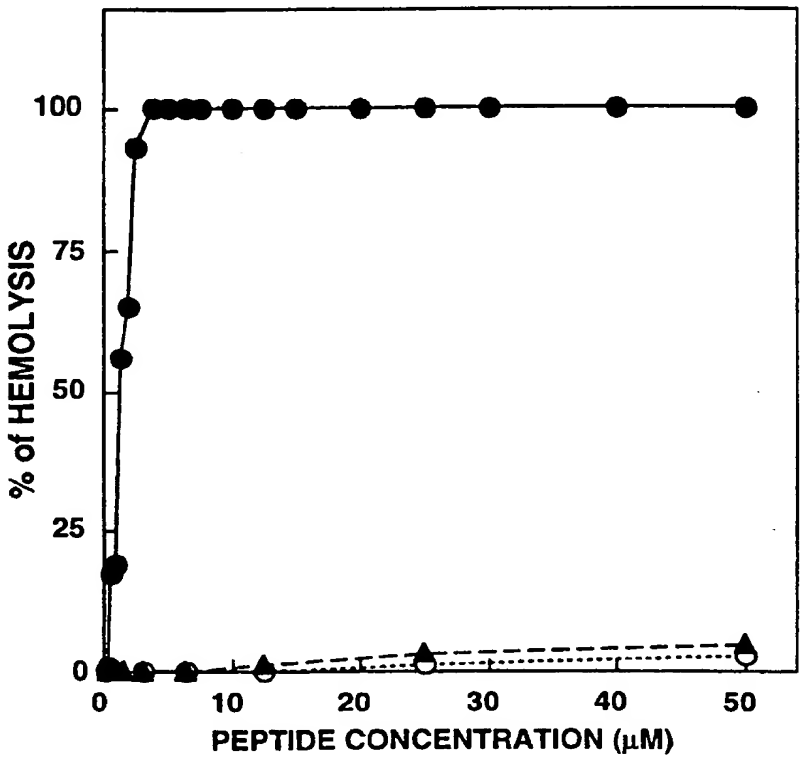
(57) Abstract

Non–hemolytic cytolytic agents selected from peptides, complexes of bundled peptides, mixtures of peptides or random peptide copolymers have a selective cytolytic activity manifested in that they have a cytolytic activity on pathogenic cells, being cells which are non–naturally occurring within the body consisting of microbial pathogenic organisms and malignant cells; and are non–hemolytic, having no cytolytic effect on red blood cells. The peptides may be cyclic derivatives of natural peptides such as pardaxin and mellitin and fragments thereof in which L–amino acid residues are replaced by corresponding D–amino acid residues, or are diastereomers of linear peptides composed of varying ratios of at least one positively charged amino acid and at least one hydrophobic amino acid, and in which at least one of the amino acid residues is a D–amino acid. Pharmaceutical compositions comprising the non–hemolytic cytolytic agents can be used for the treatment of several diseases caused by pathogens including antibacterial, fungal, viral, mycoplasma and protozoan infections and for the treatment of cancer.



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ANTIPATHOGENIC SYNTHETIC PEPTIDES AND COMPOSITIONS COMPRISING THEM

FIELD OF THE INVENTION

The present invention concerns novel non-hemolytic cytolytic agents, compositions comprising them and their use in the treatment of diseases or disorders and in agriculture.

BACKGROUND OF THE INVENTION

In the text below, reference is being made to prior art documents, the complete particulars of which can be found in the *"References"* section at the end of the specification before the claims.

The increasing resistance of microorganisms to the available antimicrobial drugs has resulted in extensive studies focused on developing alternative antimicrobial compounds.

In addition, or complementary, to the highly specific cell-mediated immune response, vertebrates and other organisms have a defense system made up of distinct groups of broad spectrum cytolytic, e.g., antibacterial, peptides.

Studies on lipid-peptide interactions of such cytolytic peptides, also known as cytolysins, tend to emphasize the importance of the amphipathic α-helical structure for their cytolytic activity. This conclusion is based mainly on studies with cytolysins that act on either mammalian cells or bacteria alone or on both types of cells. A major group of cytolytic peptides in this family are host-defense short linear peptides (≤40 amino acids), which are devoid of disulfide bridges (Boman, 1995). These peptides vary considerably in chain length, hydrophobicity and overall distribution of charges, but share a common structure upon association with lipid bilayers, namely, an amphipathic α-helix structure (Segrest et al., 1990).

Examples of known cytolysins include: (i) antibacterial peptides that are cytolytic to bacteria only, e.g. cecropins, isolated from the cecropia moth (Steiner et al., 1981), magainins (Zasloff, 1987) and dermaseptins (Mor et al., 1991) isolated from the skin of

frogs; (ii) cytolysins that are selectively cytotoxic to mammalian cells, such as δ-hemolysin isolated from *Staphylococcus aureus* (Dhople and Nagaraj, 1993); and (iii) cytolysins that are not cell-selective, such as the bee venom melittin (Habermann and Jentsch, 1967) and the neurotoxin pardaxin (Shai et al., 1988) that lyse both mammalian cells and bacteria.

Antibacterial peptides were initially discovered in invertebrates, and subsequently in vertebrates, including humans. As a complementary or additional defense system, this secondary, chemical immune system provides organisms with a repertoire of small peptides that are synthesized promptly upon induction, and which act against invasion by occasional and obligate pathogens as well as against the uncontrolled proliferation of commensal microorganisms (Boman, 1995). So far, more than 100 different antibacterial peptides have been isolated and characterized. The largest family, and probably the most studied, includes those peptides that are positively charged and adopt an amphipathic α-helical structure. Numerous studies conducted on various native antibacterial peptides tend to emphasize the importance of an amphipathic α-helical structure and a net positive charge for cytolytic activity. The positive charge facilitates interaction of the peptides with the negatively-charged membranes (Andreu et al., 1985) found in higher concentrations in the pathogenic cell membrane as compared to normal eukaryotic cells, and the amphipathic α-helical structure is essential for lytic activity (Chen et al., 1988). Such interactions have been proposed to destroy the energy metabolism of the target organism by increasing the permeability of energy-transducing membranes (Okada and Natori, 1984). Because of their amphipathic structure, it has been suggested that these antibacterial peptides permeate the membrane by forming ion channels/pores via a "barrel-stave" mechanism (Rizzo et al., 1987). According to this model transmembrane amphiphilic α-helices form bundles in which outwardly-directed hydrophobic surfaces interact with the lipid constituents of the membrane, while inwardly facing hydrophilic surfaces produce a pore. Alternatively, the peptides bind parallel to the surface of the membrane, cover the surface of the membrane in a "carpet"-like manner and dissolve it like a detergent (Shai, 1995).

Despite extensive studies, the exact mode of action of short linear non cell-selective peptides, such as pardaxin and melittin, is not known yet, and it is not clear whether similar structural features are required for their cytotoxicity towards mammalian cells and bacteria.

Pardaxin, a 33-mer peptide, is an excitatory neurotoxin that has been purified from the Red Sea Moses Sole *Pardachirus marmoratus* (Shai et al., 1988) and from the Peacock Sole of the western Pacific *Pardachirus pavoninus* (Thompson et al., 1986). Pardaxin possesses a variety of biological activities depending upon its concentration (reviewed in Shai, 1994). At concentrations below 10^{-7} M, pardaxin induces the release of neurotransmitters in a calcium-dependent manner. At higher concentrations of 10^{-7} M to 10^{-5}M, the process is calcium-independent, and above 10^{-5}M cytolysis is induced. Pardaxin also affects the activities of various physiological preparations *in vitro*. Its biological roles have been attributed to its interference with the ionic transport of the osmoregulatory system in epithelium and to presynaptic activity by forming ion channels that are voltage dependent and slightly selective to cations. A "barrel-stave" mechanism for insertion of pardaxin into membranes was proposed on the basis of its structure and various biophysical studies (reviewed in Shai, 1994). Pardaxin has a helix-hinge-helix structure: the N-helix includes residues 1-11 and the C-helix includes residues 14-26. The helices are separated by a proline residue situated at position 13. This structural motif is found both in antibacterial peptides that can act specifically on bacteria (e.g., cecropin), and in cytotoxic peptides that can lyse a variety of cells (e.g., melittin).

Melittin, a 26-mer amphipathic peptide, is the major component of the venom of the honey bee *Apis mellifera* (Habermann and Jentsch, 1967) and is one of the most studied membrane-seeking peptides (Dempsey, 1990). Melittin is highly cytotoxic for mammalian cells, but is also a highly potent antibacterial agent (Steiner et al., 1981). Numerous studies have been undertaken to determine the nature of the interaction of melittin with membranes, both with the aim of understanding the molecular mechanism of melittin-induced hemolysis and as a model for studying the general features of structures of membrane proteins and interactions of such proteins with phospholipid membranes. Much of the currently described evidence indicates that different molecular mechanisms may underlie different actions of melittin. Nevertheless, the amphipathic α-helical structure has been shown to be a prerequisite for its various activities (Perez et al., 1994).

The structure of melittin has been investigated using various techniques. The results of X-ray crystallography and NMR in methanolic solutions indicate that the molecule consists of two α-helical segments (residues 1-10 and 13-26) that intersect at an

angle of 120°. These segments are connected by a hinge (11-12) to form a bent α-helical rod with the hydrophilic and hydrophobic sides facing opposite directions. Four such monomeric melittin molecules cluster together, through hydrophobic interactions, to form a tetramer (Anderson et al., 1980; Bazzo et al., 1988; Terwilliger and Eisenberg, 1982; Terwilliger and Eisenberg, 1982). Upon initial interaction with membrane surfaces, it has been found that the tetramer dissociates to monomers, which retain α-helical conformation prior to insertion into the membrane (Altenbach and Hubbell, 1988).

Melittin shares some similarities with pardaxin. Both pardaxin and melittin are composed of two helices with a proline hinge between them. Furthermore, they exhibit significant homology in their N-helices, which are mostly hydrophobic (Thompson et al., 1986). However, pardaxin (net charge +1) contains an additional seven amino acids residue at its C-terminal side with a charge of -2, while melittin (net charge +6) terminates with an amide group and contains the positively-charged tetrapeptide sequence Lys-Arg-Lys-Arg. There are several functional differences between pardaxin and melittin. Pardaxin binds similarly to both zwitterionic and negatively charged phospholipids (Rapaport and Shai, 1991), while melittin binds better to negatively charged than to zwitterionic phospholipids (Batenburg et al., 1987; Batenburg et al., 1987). Also, pardaxin binds to phospholipids with positive cooperativity (Rapaport and Shai, 1991) while melittin binds with negative cooperativity (Batenburg et al., 1987; Batenburg et al., 1987). Although both pardaxin and melittin are potent antibacterial peptides against Gram-positive and Gram-negative bacteria, pardaxin is 40-100 fold less hemolytic than melittin towards human erythrocytes (Oren and Shai, 1996).

Analogues of pardaxin with L- to D- substitutions were shown to be capable of lysing human erythrocytes (Pouny and Shai, 1992). It was later shown (see results reported below) that two of the peptides disclosed in Pouny and Shai, 1992, namely, D-Pro^{7}-pardaxin and D-Leu^{18}Leu^{19}-pardaxin, while being hemolytic, have a very low antibacterial activity. Analogues of magainin with L- to D- substitutions were also found to lack antibacterial activity (Chen et al., 1988).

GLOSSARY

In the following, use will be made of several coined terms for the purpose of streamlining reading of the text and facilitating better understanding of the invention. It

should be noted, however, that for complete understanding of these terms, reference will at times be made to the complete description below. These terms and their meaning herein are the following:

"Heterogeneous peptide" as used herein refers to a peptide comprising both D- and L-amino acid residues.

"Homogeneous peptide" as used herein refers to a peptide comprising either only the natural L-amino acid residues, or only D-amino acid residues.

"Homogeneous L-peptide" and "homogeneous D-peptide" as used herein refers the homogeneous polypeptide consisting entirely of either L- or D-amino acid residues, respectively.

"Heterogeneous L-based peptide" and "heterogeneous D-based peptide" as used herein refers to a heterogeneous peptide comprising primarily L-amino acid residues, e.g., a peptide derived from homogeneous L-peptide in which one or more of the L-amino acid residues has been replaced by counterpart D-enantiomers, and a heterogeneous peptide comprising primarily D-amino acid residues in which one or more of the D-amino acid residues has been replaced by counterpart L-enantiomers, respectively.

"Helical peptide" as used herein refers to a peptide having a continuous α-helix stretch throughout the major portion of its length. The helical portion of a helical peptide consists entirely of either L-amino acid residues or D-amino acid residues.

"Non-helical peptide" as used herein refers to a peptide which has no α-helix structure or has non-continuous α-helix structures dispersed along its length. A non-helical peptide according to the invention may have an α-helix stretch which, in case it is terminal, has a length spanning less than half a width of a cell's membrane, e.g., less than about 10-15 amino acid residues, and if it is a non-terminal α-helix, has a length which is less than the full width of the cell's membrane, e.g., less than about 20-25 amino acid residues. A non-helical peptide may be a homogeneous peptide with α-helix breaker moieties (see below) or may be a heterogeneous peptide.

"α-helix breaker moiety" as used herein refers to a moiety which if inserted into an α-helix structure disrupts its continuity. Such a moiety may for example be the amino acid residue proline or glycine, α-methyl-substituted α-amino acids, non-α-amino acids both cyclic and acyclic such as 6-amino-hexanoic acid, 3-amino-1-cyclohexanoic acid,

4-amino-1-cyclohexanoic acid or may be an L- or D-enantiomer inserted into an α-helix stretch consisting of a stretch of amino acid residues of the opposite enantiomer.

"Pathogenic cells" as used herein refers to cells which are non-naturally occurring within the body, including cancer cells and pathogenic organisms such as bacteria, fungi, protozoa, virus and mycoplasma, as well as mammalian cells infected with pathogenic organisms such as parasitic protozoans, e.g Leishmania and Plasmodium.

"Selective cytolytic activity" as used herein refers to activity of an agent in inducing cytolysis of a pathogenic cell, the selectivity being manifested in that the agent induces cytolysis of the pathogenic cells at a much lower concentration to that required for the cytolysis of normal non-pathogenic cells such as red blood cells.

"Non-hemolytic" as used herein refers to agents which cause hemolysis of red blood cells at much higher concentrations than the concentration required to cause cytolysis of other cells, such as pathogenic cells such as microorganism cells, cancer cells, and the like.

"Diastereomers" is used herein as a synonym of "heterogeneous peptide".

SUMMARY OF THE INVENTION

The present invention provides a non-hemolytic cytolytic agent selected from a peptide, a complex of bundled peptides, a mixture of peptides or a random peptide copolymer, said agent having a selective cytolytic activity manifested in that it has a cytolytic activity on pathogenic cells, being cells which are non-naturally occurring within the body consisting of microbial pathogenic organisms and malignant cells; and it is non-hemolytic, namely it has no cytolytic effect on red blood cells or has a cytolytic effect on red blood cells at concentrations which are substantially higher than that in which it manifests said cytolytic activity, said non-hemolytic cytolytic agent being selected from the group consisting of:

(1) a cyclic derivative of a peptide having a net positive charge which is greater than +1, and comprising both L-amino acid residues and D-amino acid residues, or comprising one or both of L-amino acid residues and D-amino acid residues, and comprising an α-helix breaker moiety;

(2) a peptide comprising both L-amino acid residues and D-amino acid residues, having a net positive charge which is greater than +1, and having a sequence of

amino acids such that a corresponding amino acid sequence comprising only L-amino acid residues is not found in nature, and cyclic derivatives thereof;

(3) a complex consisting of a plurality of 2 or more non-hemolytic cytolytic peptides, each peptide having a net positive charge which is greater than +1, and comprising both L-amino acid residues and D-amino acid residues, or comprising one or both of L-amino acid residues and D-amino acid residues and comprising an α-helix breaker moiety, or cyclic derivatives of the foregoing, said peptides being bundled together by the use of a linker molecule covalently bound to each of the peptides;

(4) a mixture consisting of a plurality of 2 or more non-hemolytic cytolytic peptides, each peptide having a net positive charge which is greater than +1, and comprising both L-amino acid residues and D-amino acid residues, or comprising one or both of L-amino acid residues and D-amino acid residues and comprising an α-helix breaker moiety, or cyclic derivatives of the foregoing; and

(5) a random copolymer consisting of different ratios of a hydrophobic, a positively charged and a D-amino acid.

In one embodiment, the cyclic derivatives of (1) above are derived from a non-selective cytolytic natural peptide such as for example pardaxin and mellitin or from a fragment thereof. These cyclic diastereomers are obtained by conventional cyclization methods for peptides. In one embodiment, the cyclic diastereomer is derived from the fragment 1-22 of pardaxin to which 1 to 3 Lys residues have been added to the N-terminus and cysteine residues have been added to both N- and C-terminus for cyclization.

The net positive charge of the peptides may be due to the native amino acid composition, to neutralization of free carboxyl groups, and/or to the addition of positively charged amino acid residues or positively charged chemical groups.

In another embodiment, the invention provides a non-hemolytic cytolytic peptide and cyclic derivatives thereof as defined in (2) above, having the following characteristics:

(a) it is a non-natural synthetic peptide composed of varying ratios of at least one hydrophobic amino acid and at least one positively charged amino acid, and in which sequence at least one of the amino acid residues is a D-amino acid;

(b) the peptide has a net positive charge which is greater than +1; and

(c) the ratio of hydrophobic to positively charged amino acids is such that the peptide is cytolytic to pathogenic cells but does not cause cytolysis of red blood cells.

Examples of positively charged amino acids are lysine, arginine and histidine, and of hydrophobic amino acids are leucine, isoleucine, glycine, alanine, valine, phenylalanine, proline, tyrosine and tryptophan. The net positive charge is due to the amino acid composition, but the addition of positively charged chemical groups may also be considered. In addition, polar amino acids such as serine, threonine, methionine, asparagine, glutamine and cysteine, may be added in order to decrease the hydrophobicity and/or the toxicity of the molecule. In one preferred embodiment, the peptide is composed of one hydrophobic amino acid such as leucine, alanine or valine, and one positively charged amino acid such as lysine or arginine.The synthetic non-natural peptide may have at least 6, particularly ten or more amino amino acid residues. In one preferred embodiment, the synthetic diastereomer is a 12-mer peptide composed of leucine, alanine or valine and lysine, and at least one third of the sequence is composed of D-amino acids.

In still another embodiment, the invention provides a non-hemolytic cytolytic complex as defined in (3) above, consisting of a plurality of 2 or more non-hemolytic cytolytic peptides complexed or "bundled" together, e.g. by the use of a linker or "template" molecule covalently bound to each of the peptides. The bundle may be composed of 2 or more, preferably 5, molecules of the same peptide or of different peptides. The linker /template may be a peptide or a commonly used linker, e.g. polymers such as polyesters, polyamides, polypeptides, polyaminoacids (e.g. polylysine) carrying active groups such as OH, SH, COOH, NH_2, CH_2Br.

In still a further embodiment, the invention provides a non-hemolytic cytolytic mixture as defined in (4) above, obtained by adding a mixture composed of 1 eq each of the desired hydrophobic, positively charged and D-amino acid at each coupling step of the solid phase method for peptide synthesis. In this way, a mixture of 3^{12} different peptides were obtained with a mixture of lysine, leucine and D-leucine, and the mixture was obtained therefrom after HF cleavage, extraction with water and lyophilization.

In a further embodiment, the invention provides a non-hemolytic cytolytic random copolymer as defined in (5) above consisting of different ratios of a hydrophobic, a positively charged and a D-amino acid, e.g. 1 : 1 : 1, 2 : 1 : 1 and 3 : 1 : 1 (Mol) copolymers of Lys : Leu : D-Leu.

Preferably, the non-hemolytic cytolytic peptide has either no α-helix structure or has an α-helix structure which length is insufficient to span the width of a cell membrane.

The peptide thus does not contain an uninterrupted stretch of either all D- or all L-amino acid residues of a length capable of forming part of a transmembrane pore. Such a length is typically about 20-22 amino acids, where the stretch is in the non-terminal portion of the peptide and about half, i.e., 10-11 amino acids, where the stretch is in the terminus of the peptides, since in such a case two peptides may join their terminus together and span the cell's membrane.

The disruption of a stretch of D- or L-amino acid residues may be carried out by replacement of one or more amino acids in the stretch by the amino acid of the opposite enantiomer or by placing in the continuous stretch an α-helix breaker moiety such as proline, glycine, an α-methyl-α-amino acid or a non-α-amino acid.

The peptides of the invention and the peptides comprised within the complexes, mixtures and copolymers of the invention have a net positive charge greater than +1. The net positive charge may be due to the native amino acid composition of the invention, to neutralization of free COOH groups, for example by amidation, or may be due to addition of positively charged amino acids or chemical groups. It was found that the selective cytolytic activity can at times be enhanced by increasing the net positive charge, for example, by attaching at any position in the molecule a positively charged amino acid and/or a positively charged group. For example, a polyamine group, an alkylamino group or amino alkylamino group, etc., may be attached at one of its terminals, typically at its carboxyl terminal. A preferred such group is the aminoethylamino group -NH-CH_2-CH_2-NH_2, designated hereinafter *"TA"*.

The peptides that are derived from non-selective cytolytic natural peptides, e.g. pardaxin and melittin, are amphipathic, meaning that they have one surface which is mainly composed of hydrophobic amino acid residues and an opposite surface which is mainly composed of hydrophilic amino acid residues. The amphipathic nature of peptides may be verified according to methods known in the art. An example of such a method is the use of a Shiffer and Edmondson wheel projection wherein the amino acid residues are written, according to their sequence in a circle so that each amino acid in the sequence is angularly displaced by 100° from its neighboring amino acid residues (3.6 amino acids per circle). If most hydrophilic amino acids concentrate on one side of the wheel and hydrophobic amino acids concentrate on the opposite side of the wheel then the peptide may be considered amphipathic.

The peptides of the invention that are not derived from non-selective cytolytic natural peptides, e.g. the synthetic diastereomers composed of hydrophobic, positively charged and D-amino acids, are not amphipathic. They have a net positive charge greater than +1 and a suitable hydrophobic to positively charged amino acid ratio such that the resulting peptide is cytolytic to pathogenic cells but not hemolytic. These peptides can be screened very easily according to the invention by using the antibacterial and hemolytic tests described herein. In one embodiment, for a peptide composed of leucine and lysine, an appropriate Leu : Lys ratio may be 64% : 36% for a diastereomer of 6 amino acid residues, and 66% : 34% for a diastereomer of 12 amino acid residues

Without wishing to be bound by theory, it is believed however that the cytolytic activity may be the result of aggregation of a number of peptides on the surface of the membrane and together such peptides cause lesion of the cell membrane. Accordingly, as described above, it is contemplated in accordance with the invention also to use a plurality of peptides either as a mixture or complexed (or bundled) together, e.g., by the use of a linker molecule covalently bound to each of the peptides.

The individual peptide typically consists of at least six, and preferably ten or more amino acid residues. In a complex of the invention, each individual peptide may typically have a length of above 5 amino acid residues.

The present invention also provides a pharmaceutical composition comprising a non-hemolytic cytolytic agent of the invention as the active ingredient, and a pharmaceutically acceptable carrier. The compositions are for use in the treatment of diseases or disorders caused by different pathogenic organisms such as Gram-positive and Gram-negative bacteria, virus, fungi, mycoplasma, and parasitic protozoa, e.g Leishmania that causes leishmaniasis and Plasmodium that causes malaria. In a preferred embodiment, the anti-pathogenic composition is an antimicrobial, particularly antibacterial compositions. In addition, the compositions of the invention are useful against malignant cells and can be used in the treatment of cancer.

Also provided by the present invention is a method of treatment comprising administering said hemolytic non-cytolytic agent to a subject in need. The method of the invention as well as the above composition are applicable in both human and veterinary medicine.

Further provided in accordance with the invention is also the use of said non-hemolytic cytolytic agent in the preparation of a pharmaceutical composition for the treatment of a disease or a disorder in human or a non-human animal, in particular antibacterial compositions.

In a further embodiment, the selective agents of the invention can be used as disinfectants for the destruction of microorganisms, i.e., in solution for wetting contact lenses, may be used as preservatives, for example in the cosmetic or food industry, and as pesticides, e.g. fungicides, bactericides, in agriculture, or for preservation of agricultural products, e.g. fruits and legumes.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows circular dichroism (CD) spectra of aminoethylaminopardaxin (TApar)-derived peptides. Spectra were taken at peptide concentrations of 0.8-2.0 x 10^{-5} M in 40% 2,2,2-trifluoroethanol (TFE)/water. Symbols: TApar (——); [D]P^7–TApar (.....); [D]$L^{18}L^{19}$–TApar (——) and [D]$P^7L^{18}L^{19}$–TApar (·–·–);

Fig. 2 depicts dose-response curves of the hemolytic activity of TApar-derived peptides towards human red blood cells (hRBC). The inset shows the assay results at low concentration. Symbols: Filled squares, melittin; filled triangles, TApar; filled circles, [D]P^7-TApar; empty circles, [D]L^{18} L^{19}-TApar; empty squares, [D]P^7L^{18} L^{19}-TApar; empty triangles, dermaseptin.

Figs. 3A-B show the maximal dissipation of the diffusion potential in vesicles induced by the TApar-derived peptides. The peptides were added to isotonic K^+ free buffer containing small unilamellar vesicles (SUV) composed of egg phosphatidylcholine/phosphatidylserine (PC/PS) (Fig. 3A) or PC (Fig. 3B), pre-equilibrated with the fluorescent dye diS-C_2-5 and valinomycin. Fluorescence recovery was measured 10-20 min after the peptides were mixed with the vesicles. Symbols: Filled triangles, TApar; filled circles, [D]P^7-TApar; empty circles, [D]L^{18} L^{19}-TApar; empty squares, [D]P^7L^{18} L^{19}- TApar;

Figs. 4A-C show electron micrographs of negatively stained *E.coli* cell untreated (Fig.4A) or treated with [D]$P^7L^{18}L^{19}$–TApar at concentrations lower than the minimal inhibitory concentration (MIC) (4B) or at MIC concentrations (4C);

Fig. 5 shows CD spectra of melittin and melittin-derived diastereomers. Spectra were taken at peptide concentrations of 0.8-2.0 x 10^{-5} M in 40% TFE/water. Symbols: melittin, (——); [D]-$V^{5,8},I^{17},K^{21}$-melittin, (· · · · ·); [D]-$V^{5,8},I^{17},K^{21}$-melittin-COOH, (- - - - -).

Fig. 6 depicts dose-response curves of the hemolytic activity of the melittin-derived diastereomers towards hRBC. Symbols: filled circles, melittin; empty circles, [D]-$V^{5,8},I^{17},K^{21}$-melittin-COOH ; filled triangles, [D]-$V^{5,8},I^{17},K^{21}$-melittin.

Figs. 7A-C shows electron micrographs of negatively stained *E. coli* untreated (Fig. 7A) or treated with [D]-$V^{5,8},I^{17},K^{21}$-melittin at concentrations lower than the MIC (7B) or at the MIC concentrations (7C).

Figs. 8A-B shows maximal dissipation of the diffusion potential in vesicles induced by melittin and a melittin-derived diastereomer. The peptides were added to isotonic K^+ free buffer containing SUV composed of PC (8A) or PC/PS (8B), pre-equilibrated with the fluorescent dye diS-C_2-5 and valinomycin. Fluorescence recovery was measured 10-20 min after the peptides were mixed with the vesicles. Symbols: filled circles, melittin; filled triangles, [D]-$V^{5,8},I^{17},K^{21}$-melittin.

Figs. 9A-B show increase in the fluorescence of [D]-$V^{5,8},I^{17},K^{21}$-melittin (0.5μM total concentration) upon titration with PC/PS vesicles (filled triangles) or PC vesicles (empty triangles), with excitation wavelength set at 280 nm and emission at 340 nm. The experiment was performed at 25°C in 50 mM Na_2SO_4, 25 mM HEPES-SO_4^{-2} pH 6.8 (Fig. 9A); and binding isotherm derived from Fig. 9A by plotting X_b* (molar ratio of bound peptide per 60% of lipid) versus C_f (equilibrium concentration of free peptide in the solution) (Fig. 9B).

Figs. 10A-B show quenching of the environmentally sensitive tryptophan by brominated phospholipids. Melittin (Fig. 10A) and [D]-$V^{5,8},I^{17},K^{21}$-melittin (10B) were added to buffer containing PC/PS (1:1 w/w) SUV. The SUV contained 25% of either 6,7 Br-PC (_ · _ · _ · _), or 9,10 Br-PC (- - - -), or 11,12 Br-PC (· · · · ·). After 2 min incubation, an emission spectrum of the tryptophan was recorded using spectrofluorometer

with excitation set at 280 nm. For comparison PC/PS (1:1 w/w) SUV with no Br-PC were used (_____).

Fig. 11 shows the effect of the hydrophobicity of the Leu/Lys diastereomers on RP-HPLC retention time.

Fig. 12 shows dose-response curves of the hemolytic activity of the Leu/Lys diastereomers towards hRBC. The inset shows the assay results at low concentrations. Symbols : empty squares, melittin; filled squares, [D]-$L^{3,4,8,10}$-K_3 L_9; filled circles, [D]-$L^{3,4,8,10}$-K_4L_8; empty triangles, [D]-$L^{3,4,8,10}$-K_5L_7; filled triangles, [D]-$L^{3,4,8,10}$-K_7L_5.

Figs. 13A-B show maximal dissipation of the diffusion potential in vesicles, induced by the Leu/Lys diastereomers . The peptides were added to isotonic K^+ free buffer containing SUV composed of PC (Fig. 13A) or PE/PG (13B), pre-equilibrated with the fluorescent dye diS-C_2-5 and valinomycin. Fluorescence recovery was measured 3-10 min after the peptides were mixed with the vesicles. Symbols: filled squares, [D]-$L^{3,4,8,10}$-K_3 L_9; filled circles, [D]-$L^{3,4,8,10}$-K_4L_8; filled triangles, [D]-$L^{3,4,8,10}$-K_5L_7; crossed circles, [D]-$L^{3,4,8,10}$-K_7L_5.

Figs. 14A-H show electron micrographs of negatively stained *E. coli* untreated and treated with the various Leu/Lys diastereomers at 80% of their MIC. Fig. 14A, control; Fig. 14B, *E. coli* treated with[D]-$L^{3,4,8,10}$-K_3 L_9; Fig. 14C, *E. coli* treated with [D]-$L^{3,4,8,10}$-K_4L_8 ; Fig. 14D, *E. coli* treated with [D]-$L^{3,4,8,10}$-K_5L_7; Fig. 14E, *E. coli* treated with [D]-$L^{3,4,8,10}$-K_7L_5; Fig. 14F, control; Fig. 14G, *E. coli* treated with [D]-$L^{3,4,8,10}$-K_4L_8; Fig. 14H, *E. coli* treated with [D]-$L^{3,4,8,10}$-K_5L_7.

DETAILED DESCRIPTION OF THE INVENTION

Heterogeneous L-based peptides have been found by the present inventors to possess selective cytolytic activity manifested by a selective destruction of pathogenic cells, e.g., bacteria, with little or no effect on non-pathogenic cells, i.e., red blood cells. This finding is very surprising in view of the prevalent belief in the art that the cytolytic

activity of cytolytic peptides in cells, whether pathogenic cells such as bacteria or normal mammalian cells, arises from a single underlying mechanism associated with the α-helix configuration.

Functional and structural studies with D-amino acid incorporated analogues (diastereomers) of pardaxin and melittin, two known non-cell selective cytolysins, carried in order to understand the molecular mechanism underlying cell selectivity, revealed that the resulting diastereomers did not retain their α-helical structure, which caused abrogation of their cytotoxic effects on mammalian cells. However, the diastereomers retained a high antibacterial activity, which was expressed by complete lysis of both Gram-positive and Gram-negative bacteria. Thus, the α-helical structure of pardaxin and meliittin was shown to be important for cytotoxicity against mammalian cells, but not to be a prerequisite for antibacterial activity. However, in another study, a single D-amino acid incorporated into the non-hemolytic antibacterial peptide magainin abolished almost totally its antibacterial activity (Chen et al., 1988). The results with pardaxin and melittin diastereomers suggest that hydrophobicity and a net positive charge confer selective antibacterial activity to non-selective cytolytic peptides and that amphipathic α-helical structure is not required. However, the diastereomers of pardaxin and melittin contained long stretches of L-amino acids (14-17 aa long) which raises the possibility that the low residual helicity could be sufficient for membrane binding and destabilization.

To examine whether modulating hydrophobicity and the net positive charge of linear cytotoxic peptides is sufficient to confer selective antibacterial activity, we chose to investigate diastereomers of short model peptides (12 aa. long), composed of varying ratios of leucine and lysine and one third of their sequence composed of D-amino acids. Peptide length and the position of D-amino acids were such that short peptides with very short consecutive stretches of 1-3 L-amino acids that cannot form an α-helical structure were constructed. The diastereomers were evaluated with regard to (1) their cytotoxicity against bacteria and human erythrocytes, (2) their structure, and (3) their ability to interact and perturb the morphology of the bacterial wall and model phospholipid membranes. The data show that modulating hydrophobicity and positive charge is sufficient to confer antibacterial activity and cytolytic selectivity. Furthermore, the resulting antibacterial peptides act synergistically at non lethal concentrations with available antibacterial drugs such as tetracycline, and they are totally resistant to human serum inactivation which

dramatically reduces the activity of native antibacterial peptides. Further shorter diastereomers (6 aa and 8 aa long) were prepared and tested and found to be non-hemolytic cytolytic.

The finding that certain cytolytic non-helical peptides have an anti-pathogenic activity, paves the way for the preparation of anti-pathogenic agents, which comprise such non-helical polypeptides. Where the non-helical peptides are heterogeneous peptides composed of both L-amino acids and D-amino acids, the anti-pathogenic agents have the additional advantage of being more resistant to degradation, for example by proteases, than homologous L-peptides, on the one hand, and on the other hand, are not completely degradation-resistant as the full homologous D-peptides. Resistance to degradation may be disadvantageous in view of slow clearance from the body with possible associated toxic side effects. The non-α-helical antipathogenic peptides may be used in a variety of therapeutic procedures.

Since it is known that homologous D-peptides possess essentially identical cytolytic activity to the corresponding homologous L-peptides (Bessalle et al., 1990) then accordingly it is clear that heterogeneous D-based peptides possess the same antipathogenic properties as heterogeneous L-based peptides.

The finding that certain non-α-helical peptides have a cytolytic activity against bacteria without a cytolytic activity against red blood cells, is a result of the fact that bacterial cells differ from red blood cells in the composition of their cell membrane. Differences in the composition of the cell membrane can also be found among a variety of pathogenic cells, such as cancer cells, and normal cells. Thus, based on this finding, the agents of the present invention pave the way for development of a variety of drugs having a selective cytolytic activity against one class of cells within the body such as bacteria cells, cells of a parasite, fungus cells, protozoa cells, or cancer cells, with little or no activity against non-pathogenic normal body cells.

The non-hemolytic cytolytic agents the invention having a selective cytolytic activity against pathogenic cells, while having a much lower, or no cytolytic activity against normal, non-pathogenic cells, may be used for a variety of therapeutic applications with no or little toxic side effects.

One group of cyclic peptides in accordance with the invention are derived from non-α-helical heterogeneous peptides derived from homogeneous peptides with an

α-helical structure possessing a broad range cytolytic activity. The present invention thus provides in accordance with one embodiment, a heterogeneous peptide cyclic derivative comprising both D- and L-amino acid residues having a sequence such that a homogeneous open chain peptide comprising only L- or only D-amino acid residues and having the same amino acid sequence as said heterogeneous peptide, has an α-helix configuration and has a broad spectrum cytolytic activity manifested on a variety of cells; said heterogeneous cyclic peptide having a cytolytic activity on only some of the cells on which said homogeneous peptide is cytolytically active. For example, a cytolytic activity of the heterogeneous cyclic peptide is manifested only on pathogenic cells while having no cytolytic activity on normal cells such as red blood cells.

Examples of non-hemolytic cytolytic cyclic peptides in accordance with the invention are such which are derived from natural peptides which have an α-helical structure and possess a cytolytic activity. The non-α-helical cyclic peptides of the invention have a sequence essentially corresponding to the entire or partial sequence of the natural peptide in which D-amino acids are incorporated along the N- and C-helices of the molecule and a net positive charge is attained either by addition of a positively charged amino acid residue, e.g., lysine, arginine, histidine, for example at the N-terminus and/or of a positively charged group, e.g. aminoalkylamino group such as aminoethylamino, for example at the C-terminus of the molecule, or by neutralization of free carboxyl groups e.g. by converting them to amide groups. Examples of such natural peptides are melittin and pardaxin, and fragments thereof.

For example, the non-α-helical cyclic peptide may be derived from pardaxin which is a 33-mer peptide or from melittin, which is a 26-mer peptide, the non-α-helical cyclic peptide may be a 33-mer or a 26-mer peptide comprising a sequence corresponding to the entire sequence of pardaxin or of melittin, respectively, or may be a non-helical cyclic peptide having a sequence corresponding to a partial sequence of pardaxin or melittin, e.g., 8-23 mer melittin sequence. In the case of a heterogeneous cyclic peptide derived from pardaxin, the heterogeneous cyclic peptide in accordance with the invention may comprise a partial sequence corresponding to that of pardaxin, comprising as little as 10 amino acid residues and ranging between 10 and 24 amino acid residues.

Another group of peptides in accordance with the invention are non-helical peptides which have a sequence having no natural homologs and are composed of at least

one hydrophobic and at least one positively charged amino acid and in which sequence at least one amino acid residue is a D-amino acid.

Previous studies with model peptides used to elucidate the structure-function study of antibacterial peptides focused on three parameters; helical structure, hydrophobicity and charge (Anzai et al., 1991; Agawa et al., 1991). Each change in one of these parameters simultaneously resulted in changes in the other two, making it difficult to clarify the unique contribution of each parameter to the overall antibacterial activity. According to the present invention, the effect of the helical structure was eliminated which therefore permitted the study of only two parameters, namely, hydrophobicity and net positive charge, by varying the ratio of leucine and lysine. For this purpose, diastereomers of short model peptides (12 aa. long) containing stretches of only 1-3 consecutive L-amino acids which are too short to form an a-helical structure, were chosen for investigation.

CD spectroscopy revealed that these Leu/Lys diastereomers are indeed totally devoid of α-helical structure (data not shown), unlike the diastereomers of melittin and pardaxin of the invention which retain low α-helical structure. Nevertheless, the Leu/Lys diastereomers exhibit potent antibacterial activity similar to or greater than that of native antibacterial peptides such as dermaseptin S, or the antibiotic drug tetracycline. Moreover, the most potent peptides [D]-$L^{3,4,8,10}$-K_4L_8 and [D]-$L^{3,4,8,10}$-K_5L_7 (peptides **23** and **24**, respectively, of Example 3 herein) were devoid of hemolytic activity against the highly cytolytically-susceptible human erythrocytes. It should be noted that [D]-$L^{3,4,8,10}$-K_3L_9 (peptides **22**) is devoid of α-helical structure but has considerable hemolytic activity which approaches that of the native cytolytic peptide, pardaxin. This could indicate that the balance between hydrophobicity and positive charge compensates for the amphipathic α-helical structure. However, increasing the positive charge drastically reduced the hemolytic activity while antibacterial activity was preserved, demonstrating that the amphipathic α-helical structure is not required for antibacterial activity.

The interaction of the Leu/Lys diastereomers with both negatively-charged and zwitterionic phospholipid membranes was examined in order to elucidate the basis of their selective cytotoxicity against bacteria. Negatively-charged PE/PG vesicles were used to mimic the lipid composition of *E. coli* (Shaw, 1974), and the zwitterionic PC vesicles to mimic the outer leaflet of human erythrocytes (Verkleij et al., 1973). The biological activity of the Leu/Lys peptides on erythrocytes (Fig. 12) and *E. coli* (Table 5) correlates

well with their ability to permeate model membranes. The only peptide which permeated PC vesicles was the only peptide with significant hemolytic activity. These results suggest that the phospholipid composition of the bacterial membrane plays a role in permeation by this family of antibacterial peptides. The ability of antibacterial and non-hemolytic peptides to bind and permeate negatively-charged but not zwitterionic phospholipid vesicles is characteristic of native antibacterial peptides (Gazit et al., 1994), and has been attributed to the fact that the bacterial surface contains lipopolysaccharides (LPS, in Gram-negative bacteria), and polysaccharides (teichoic acids, in Gram-positive bacteria), and their inner membranes contain phosphatidyl glycerol (PG), all of which are negatively charged, while normal eukaryotic cells such as erythrocytes, predominantly express the zwitterionic phospholipid PC on their outer leaflet.

The antibacterial peptide magainin is a non-hemolytic peptide, while melittin, pardaxin, and a model peptide with a sequence similar to that of [D]-$L^{3,4,8,10}$-K_4L_8, but composed of entirely L-amino acids, are hemolytic, mainly due to their high hydrophobicity. When the α-helical structure of magainin was disrupted by the introduction of three D-amino acids, the resulting diastereomer had no antibacterial activity (Chen et al.,1988), even though its net positive charge is similar to that of native magainin. Thus, an optimal balance that already exists between the α-helical structure, hydrophobicity and net positive charge of native magainin, allows selective antibacterial activity, and any change in one of these properties could cause a loss in magainin's antibacterial activity. Contrastingly, hydrophobicity appears to play a major role in compensating for the loss of α-helical structure in melittin, pardaxin and the Leu/Lys diastereomers of the invention.

The results according to the invention suggest a new strategy for the design of a repertoire of short, simple, and easily manipulated antibacterial peptides. Each of the diastereomeric model Leu/Lys peptides has a unique spectrum of activity (Table 5). The existence of a repertoire of diastereomeric antibacterial peptides will enable one to choose the most efficacious peptide with regard to the target cell. Furthermore, simultaneous administration of multiple forms of diastereomers peptides, acting separately or in concert, also has a selective survival value, and provides a better shielding against a wider range of infectious microbes. All the Leu/Lys diastereomers displayed increased antibacterial activity against Gram-positive in comparison to Gram-negative bacteria. These results are

important considering the increasing resistance of Gram-positive bacteria such as *Staphylococcus aureus, enterococci*, and *pneumococci* to conventional antibiotics (Russell et al., 1995). In addition, unlike the native antibacterial peptide dermaseptin S, [D]-$L^{3,4,8,10}$-K_5L_7 (peptide **24**) retained its antibacterial activity in the presence of pooled human serum.

Diastereomeric peptides should have several advantages over known antibacterial peptides: (1) The peptides should lack the diverse pathological and pharmacological effects induced by α-helical lytic cytolysins. For example, staphylococcus δ-toxin, the antibacterial peptide alamethicin, cobra direct lytic factor and pardaxin exert several histopathological effects on various cells due to pore formation and activation of the arachidonic acid cascade. However, pardaxin diastereomers do not exert these activities. In addition, many amphipathic α-helical peptides bind to calmodulin and elicit several cell responses, and even all D-amino acid α-helices, including melittin have similar activity (Fisher et al., 1994). Diastereomers with disrupted α-helical structure are not expected to bind to calmodulin; (2) Local D-amino acid substitution would result in controlled clearance of the antibacterial peptides by proteolytic enzymes, as opposed to the total protection acquired by complete D-amino acids substitution (Wade et al., 1990). Total resistance of a lytic peptide to degradation is disadvantageous for therapeutic use. Furthermore, the antigenicity of short fragments containing D,L amino acids is dramatically altered as compared to their wholly L or D-amino acid parent molecules (Benkirane et al., 1993); (3) Total inhibition of bacterial growth induced by the diastereomers, is associated with total lysis of the bacterial wall, as shown by electron microscopy (Fig. 14). Therefore, bacteria might not easily develop resistance to drugs that trigger such a destructive mechanism; (4) [D]-$L^{3,4,8,10}$-K_5L_7 (peptide **24**) has the ability to perturb the cell wall of bacteria at concentrations lower than their MIC, as seen by electron microscopy (Fig. 14). The simultaneous administration of clinically used antibiotics, which have no activity due to their inability to penetrate the bacterial cell wall, together with peptide **24,** may present a solution to this resistance mechanism of bacteria.

The invention will now be described with reference to some non-limiting drawings and examples.

EXPERIMENTAL PROCEDURES

(i) Materials. Butyloxycarbonyl-(amino acid)-(phenylacetamido) methyl resin was purchased from Applied Biosystems (Foster City, CA) and butyloxycarbonyl (Boc) amino acids were obtained from Peninsula Laboratories (Belmont, CA). Other reagents used for peptide synthesis included trifluoroacetic acid (TFA, Sigma), N,N-diisopropylethylamine (DIEA, Aldrich, distilled over ninhydrin), dicyclohexylcarbodiimide (DCC, Fluka), 1-hydroxybenzotriazole (HOBT, Pierce) and dimethylformamide (peptide synthesis grade, Biolab). Egg phosphatidylcholine (PC) and phosphatidylserine (PS) from bovine spinal cord (sodium salt-grade I) were purchased from Lipid Products (South Nutfield, U.K). Egg phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) (Type V, from *Escherichia coli*) were purchased from Sigma. Cholesterol (extra pure) was supplied by Merck (Darmstadt, Germany) and recrystallized twice from ethanol. 3,3'-Diethylthio-dicarbocyanine iodide [diS-C_2-5] was obtained from Molecular Probes (Eugene, OR). Native melittin was purchased from Sigma. Commercially available melittin usually contains traces of phospholipase A_2, which causes rapid hydrolysis of phospholipids. Therefore, special care was taken to remove all the phospholipase A_2 from melittin using RP-HPLC. All other reagents were of analytical grade. Buffers were prepared in double glass-distilled water.

(ii) Peptide synthesis and purification. Peptides were synthesized by a solid phase method on butyloxycarbonyl-(amino acid)-(phenylacetamido) methyl resin (0.05 meq) (Merrifield et al., 1982). The resin-bound peptides were cleaved from the resins by hydrogen fluoride (HF), and after HF evaporation extracted with dry ether. These crude peptide preparations contained one major peak, as revealed by RP-HPLC, that was 50-70% pure peptide by weight. The synthesized peptides were further purified by RP-HPLC on a C_{18} reverse phase Bio-Rad semi-preparative column (300Å pore size). The column was eluted in 40 min, using a linear gradient of 10-60% acetonitrile in water, both containing 0.05% TFA (v/v), at a flow rate of 1.8 ml/min. The purified peptides, which were shown to be homogeneous (~95%) by analytical HPLC, were subjected to amino-acid analysis and to mass spectrometry to confirm their sequences.

(iii) Transamination of the peptides. The resin-bound peptides as in (ii) above were transaminated with 30% ethylene diamine in DMF for 3 days, followed by filtration of the resin, precipitation of the protected peptides, namely aminoethylamino (TA) peptides, with

ether and removal of the protecting groups with HF. The synthetic TA-peptides were purified (>95% homogenicity) by reverse-phase HPLC on a C_{18} column using a linear gradient of 25-80% acetonitrile in 0.1% TFA, in 40 min, and then subjected to amino acid analysis to confirm their composition.

(iv) Amidation of the peptides. Resin-bound peptide (20 mg) was treated for 3 days with a mixture composed of 1:1 v/v of saturated ammonia solution (30%) in methanol and DMSO (1:1 v/v) which resulted in amidation of the carboxylate group of the glutamine residue located at the C-terminus of [D]-$V^{5,8},I^{17},K^{21}$-melittin. Thus, peptides were obtained in which all the protecting groups remained attached, but whose C-terminal residues were modified by one amide group. The methanol and ammonia were evaporated under a stream of nitrogen, and the protected peptides were extracted from the resin with DMSO, and precipitated with dry ether. The products were then subjected to HF cleavage and to further purification using RP-HPLC as described above.

(v) Preparation of lipid vesicles. Small unilamellar vesicles (SUV) were prepared by sonication of PC/cholesterol (10:1 w/w) or PC/PS (1:1 w/w) dispersions. Briefly, dry lipid and cholesterol (10:1 w/w) were dissolved in a $CHCl_3$/MeOH mixture (2:1 v/v). The solvents were then evaporated under a stream of nitrogen and the lipids (at a concentration of 7.2 mg/ml) were subjected to a vacuum for 1 h and then resuspended in the appropriate buffer, by vortexing. The resultant lipid dispersions were then sonicated for 5-15 min in a bath type sonicator (G1125SP1 sonicator, Laboratory Supplies Company Inc., NY) until clear. The lipid concentrations of the resulting preparations were determined by phosphorus analysis (Bartlett, 1959). Vesicles were visualized using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan) as follows. A drop of vesicles was deposited on a carbon-coated grid and negatively stained with uranyl acetate. Examination of the grids demonstrated that the vesicles were unilamellar with an average diameter of 20-50 nm (Papahadjopoulos and Miller, 1967).

(vi) Preparation of serum. Blood was collected from five volunteers and allowed to clot at room temperature for 4 h. The blood was then centrifuged for 15 min at 1500 *g*, and the serum was removed and pooled. The serum complement was inactivated by heating at 56° C for 30 min.

(vii) CD Spectroscopy. The CD spectra of the peptides were measured with a Jasco J-500A spectropolarimeter after calibrating the instrument with (+)-10-camphorsulfonic

acid. The spectra were scanned at 23°C in a capped, quartz optical cell with a 0.5 mm path length. Spectra were obtained at wavelengths of 250 to 190 nm. Eight scans were taken for each peptide at a scan rate of 20 nm/min. The peptides were scanned at concentrations of 1.5×10^{-5} - 2.0×10^{-5} M in 40% trifluoroethanol (TFE), a solvent that strongly promotes α-helical structure. Fractional helicities (Greenfield and Fasman, 1969; Wu et al., 1981) were calculated as follows:

$$f_h = \frac{[\theta]_{222} - [\theta]^0_{222}}{[\theta]^{100}_{222} - [\theta]^0_{222}}$$

where $[\theta]_{222}$ is the experimentally-observed mean residue ellipticity at 222 nm, and the values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, which correspond to 0% and 100% helix content at 222 nm, are estimated to be 2000 and 32000 deg·cm^2/dmole, respectively (Wu et al., 1981).

(viii) Antibacterial activity of the peptides. The antibacterial activity of the diastereomers was examined in sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 100 µL as follows: Aliquots (50µl) of a suspension containing bacteria at a concentration of 10^6 Colony-Forming Units (CFU)/ml LB (Lauria broth) medium were added to 50 µL of water or 66% pooled normal human serum in PBS, containing the peptide in 2-fold serial dilutions. Growth inhibition was determined by measuring the absorbance at 492 nm with a Microplate autoreader El309 (Bio-tek Instruments), following incubation for 18-20 h at 37°C. Antibacterial activity is expressed as the minimal inhibitory concentration (MIC), the concentration at which 100% inhibition of growth was observed after 18-20 h of incubation. The bacteria used were: *Escherichia coli* D21, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter calcoaceticus* Ac11, *Salmonella typhimurium* LT2, *Bacillus megaterium* Bm11, *Micrococcus luteus* ATCC 9341, *Bacillus subtilis* ATCC 6051.

(ix) Hemolysis of human red blood cells. The peptides were tested for their hemolytic activities against human red blood cells (hRBC). Fresh hRBC with EDTA were rinsed 3 times with PBS (35 mM phosphate buffer/0.15 M NaCl, pH 7.3) by centrifugation for 10 min at 800g and resuspended in PBS. Peptides dissolved in PBS were then added to 50µL of a solution of the stock hRBC in PBS to reach a final volume of 100 µL (final erythrocyte concentration, 5% v/v). The resulting suspension was incubated under agitation for 30 min at 37°C. The samples were then centrifuged at 800g for 10 min.

Release of hemoglobin was monitored by measuring the absorbance of the supernatant at 540 nm. Controls for zero hemolysis (blank) and 100% hemolysis consisted of hRBC suspended in PBS and Triton 1%, respectively.

(x) Visualization of the effects of the peptides on bacteria by electron microscopy. Samples containing *E. coli* (10^6 CFU/ml) in LB medium were incubated with the various peptides at their MIC, and one dilution less than the MIC, for 16 h, and then centrifuged for 10 min at 3000g. The pellets were resuspended and a drop containing the bacteria was deposited onto a carbon-coated grid which was then negatively-stained with 2% phosphotungstic acid (PTA), pH 6.8. The grids were examined using a JEOL JEM 100B electron microscope.

(xi) Membrane permeation induced by the peptides. Membrane permeation was assessed utilizing the diffusion potential assay (Loew et al., 1983; Sims et al., 1974) as previously described (Shai et al., 1991). In a typical experiment, in a glass tube, 4 µl of a liposomes suspension (final phospholipids concentration of 33 µM), in a K^+ containing buffer (50 mM K_2SO_4, 25 mM HEPES-SO_4^{-2}, pH 6.8), was diluted in 1 ml of an isotonic K^+ free buffer (50 mM Na_2SO_4, 25 mM HEPES-SO_4^{-2}, pH 6.8), and the fluorescent, potential-sensitive dye diS-C_2-5 was then added. Valinomycin (1 µl of 10^{-7} M) was added to the suspension in order to slowly create a negative diffusion potential inside the vesicles, which led to a quenching of the dye's fluorescence. Once the fluorescence had stabilized, which took 3-10 minutes, peptides were added. The subsequent dissipation of the diffusion potential, as reflected by an increase in fluorescence, was monitored on a Perkin Elmer LS-50B spectrofluorometer, with the excitation set at 620 nm, the emission at 670 nm, and the gain adjusted to 100%. The percentage of fluorescence recovery, F_t, was defined as:

$$F_t = (I_t - I_0 / I_f - I_0) \times 100$$

where I_0 = the initial fluorescence, I_f = the total fluorescence observed before the addition of valinomycin, and I_t = the fluorescence observed after adding the peptide at time t.

(xii) Binding of peptides to vesicles. The interaction of [D]-$V^{5,8}$,I^{17},K^{21}-melittin with vesicles consisting of zwitterionic (PC) or negatively charged phospholipids (PC/PS) was characterized by measuring changes in the emission intensity of the peptides' intrinsic tryptophan in SUV titration experiments. Briefly, SUV were added to a fixed amount of

peptide (0.5 μM) dissolved in buffer containing 50 mM Na_2SO_4, 25 mM HEPES-SO_4^{-2}, pH 6.8, at 24°C. A 1-cm pathlength quartz cuvette that contained a final reaction volume of 2 ml was used in all experiments. The fluorescence intensity was measured as a function of the lipid/peptide molar ratio (4 separate experiments) on a Perkin-Elmer LS-5 Spectrofluorometer, with excitation set at 280 nm, using a 5 nm slit, and emission set at 340 nm, using a 2.5 nm slit. The binding isotherms were analyzed as a partition equilibrium, using the following formula:

$$X_b = K_P C_f$$

where X_b is defined as the molar ratio of bound peptide (C_b) per total lipid (C_L), K_P corresponds to the partition coefficient, and C_f represents the equilibrium concentration of the free peptide in solution. For practical purposes, it was assumed that the peptides initially were partitioned only over the outer leaflet (60%) of the SUV. Therefore, the partition equation becomes:

$$X_b^* = K_P^* C_f$$

where X_b^* is defined as the molar ratio of bound peptide per 60% of total lipid and K_P^* is the estimated surface partition constant. The curve resulting from plotting X_b^* vs. free peptide, C_f is referred to as the conventional binding isotherm.

(xiii) Tryptophan quenching experiments. Tryptophan which is sensitive to its environment has been utilized previously in combination with brominated phospholipids (Br-PC) to evaluate peptide localization in the membrane (Bolen and Holloway, 1990; De Kroon et al., 1990). Br-PC employed as quenchers of tryptophan fluorescence are suitable for probing the membrane insertion of peptides, since they act over a short distance and do not drastically perturb the membrane. Melittin and its diastereomer, each of which contains one tryptophan residue, were added (final concentration of 0.5 μM) to 2 ml of buffer (50 mM Na_2SO_4, 25 mM HEPES-SO_4^{-2}, pH 6.8) containing 20 μl (50 μM) of Br-PC/PS (1:1 w/w) SUV, thus establishing a lipid /peptide ratio of 100:1. After a 2 min incubation at room temperature, an emission spectrum of the tryptophan was recorded using a Perkin-Elmer LS-50B Spectrofluorometer, with excitation set at 280 nm (8 nm slit). SUV composed of PC/PS (1:1 w/w) and which contained 25 % of either 6,7 Br-PC,or 9,10 Br-PC, or 11,12 Br-PC, were used. Three separate experiments were

conducted for each peptide. In control experiments, PC/PS (1:1 w/w) SUV without Br-PC were used.

EXAMPLE 1. Synthesis and biological activity of pardaxin-derived diastereomers

1.1 Synthesis. To examine the role of the α-helical structure of a polycationic cytolysin in its cytotoxicity towards mammalian cells and bacteria, a series of pardaxin-derived peptides were synthesized as described in sections (ii) and (iii) of the Experimental Procedures, and characterized for their structure, hemolytic activity on hRBCs, antibacterial activity and effect on the morphology of bacteria.

Pardaxin (par) is a 33-mer peptide of the following sequence:

Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-
Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-
Gly-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Gly-Gln-Glu

Modification of the pardaxin molecule in order to introduce a positive charge was made by either deleting the acidic C-terminus of pardaxin or converting the acidic C-terminus of pardaxin or of a fragment thereof to a positive one by reaction of both carboxyl groups of the Glu residue at the C-terminus with ethylene diamine (TA), and/or adding positively charged amino acid residues such as Lys to the N-terminus, in pardaxin diastereomers in which the N-helix and/or the C-helix were altered by either replacing the residue Pro at position 7 of TApar or of a pardaxin fragment by D-Pro (herein indicated by $[D]P^7$), or the two Leu residues at positions 18 and 19 of TApar or of a pardaxin fragment by D-Leu (herein $[D]L^{18}\ L^{19}$), or both (herein $[D]P^7\ L^{18}L^{19}$). The D-amino acids were introduced in the centers of the N- and C-helices.

The following pardaxin-derived diastereomers were found to be non-hemolytic and to exhibit selective cytolytic activity (the bold and underlined residues are D-amino acids). The peptides will be represented hereinafter by numerals in bold.

1. $[D]P^7\ L^{18}L^{19}$ -TApar of the sequence:

Gly-Phe-Phe-Ala-Leu-Ile-**<u>Pro</u>**-Lys-Ile-Ile-Ser-
Ser-Pro-Leu-Phe-Lys-Thr-**<u>Leu-Leu</u>**-Ser-Ala-Val-
Gly-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Gly-Gln-Glu-$(NH\text{-}CH_2\text{-}CH_2\text{-}NH_2)_2$

2. [D]P^7 $L^{18}L^{19}$ [1-22]-TApar of the sequence:

Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-Ser-
Ser-Pro-Leu-Phe-Lys-Thr-**Leu-Leu**-Ser-Ala-Val-NH-CH_2-CH_2-NH_2

3. [D]P^7 $L^{18}L^{19}$ [1-22]-par of the sequence:

Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-Ser-
Ser-Pro-Leu-Phe-Lys-Thr-**Leu-Leu**-Ser-Ala-Val

4. K^1 [D]P^7 $L^{18}L^{19}$ [1-22]-TApar of the sequence:

Lys-Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-Ser-
Ser-Pro-Leu-Phe-Lys-Thr-**Leu-Leu**-Ser-Ala-Val-NH-CH_2-CH_2-NH_2

5. K^1 K^2[D]P^7 $L^{18}L^{19}$ [1-22]-TApar of the sequence:

Lys-Lys-Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-Ser-
Ser-Pro-Leu-Phe-Lys-Thr-**Leu-Leu**-Ser-Ala-Val-NH-CH_2-CH_2-NH_2

6. K^1 K^2[D]P^7 $L^{18}L^{19}$ [1-22]-par of the sequence:

Lys-Lys-Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-Ser-
Ser-Pro-Leu-Phe-Lys-Thr-**Leu-Leu**-Ser-Ala-Val

7. [D]P^7-[1-11]-TApar of the sequence:

Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-Ser-NH-CH_2-CH_2-NH_2

The following pardaxin derivatives were synthesized and found to be hemolytic as shown in Table 1 hereinafter:

8. TApar
9. [D]P^{13}-TApar
10. [D] L^5L^{19} -TApar
11. [D]P^7L^{19} -TApar
12. [D]P^7-TApar
13. [D]P^7-par
14. [D] $L^{18}L^{19}$ -TApar
15. [D] $L^{18}L^{19}$ -par
16. [D]P^7 $L^{18}L^{19}$ -par
17. [D]P^7 [1-22]-TApar

1.2 Determination of the secondary structure of the peptides. The secondary structures of the peptides **1, 8, 12, 14,** were evaluated from their CD spectra in 40% TFE, a solvent that strongly promotes an α-helical structure, as described in Experimental Procedures, section (vii), and in PBS (35 mM phosphate buffer/0.15 M NaCl, pH 7.0).

The CD spectra of the pardaxin-derived diastereomers are shown in Fig. 1 wherein **[8]** (___), **[12]** (.....), **[14]** (-----), and **[1]** (-.-- -.-.). As expected, a dramatic decrease in the α-helix content of the peptides was observed as more D-amino acids were incorporated, as reflected by the minima at 208 and 222 nm in 40% TFE. There was a more than 90% decrease in the α-helix content between **8** (TApar) (50% α-helix) and **1** ([D]$P^7L^{18}L^{19}$-TApar) (4%). The α-helix contents of **12** ([D]P^7-TApar) and **14** ([D]L^{18} L^{19}-TApar) were 25% and 15%, respectively. It should be noted that proline at position 7 does not introduce a kink in the structure but rather participates in the formation of the N-helix as revealed by NMR spectroscopy (Zagorski et al., 1991). In PBS, pardaxin gave a low value of ~12% α-helix content while all the analogues with D-amino acid residues gave very low signals that could not be attributed to specific structures (data not shown).

1.3 Hemolytic and antibacterial activity. The pardaxin-derived peptides **1-17** were then examined for their hemolytic activity towards the highly susceptible human erythrocytes, and for their potential to inhibit the growth of different species of bacteria, as described in Experimental Procedures, sections (ix) and (xviii), respectively. In addition, the cytotoxic bee venom melittin, the antibacterial peptide dermaseptin S, and the antibiotic tetracycline were used as controls.

Fig. 2 shows the dose response curves of the hemolytic activity of the peptides **1, 8, 12, 14**. It is shown that D-amino acids introduced into TApar dramatically reduced its hemolytic activity, which correlates with the loss of α-helix content in the corresponding analogues. Peptide **8,** TApar, with the highest α-helix content is the most hemolytic, while Peptide **1,** [D]$P^7L^{18}L^{19}$-TApar, with the lowest α-helix content, is practically devoid of hemolytic activity up to the maximum concentration tested (50 μM). The inability to lyse RBCs is characteristic of most of the naturally occurring antibacterial peptides such as dermaseptin (see Fig. 2), magainin and cecropins.

Table 1 gives the MIC (in μM) of the peptides **1-17** for a representative set of test bacteria, which includes two Gram-negative species, *Escherichia coli* and *Acinetobacter calcoaceticus*, and two Gram-positive species, *Bacillus megaterium* and *Bacillus subtilis*, as well as the % hemolysis at 50 μM peptide. Table 2 gives the MIC (in μM) of the peptides **1, 8, 12, 14** and of melittin, dermaseptin S and tetracycline for some bacterial species The data reveal that despite the dramatic decrease in the α-helix content and hemolytic activity of the diastereomeric analogues **1-7**, they all retained most of the potent antibacterial activity of the parent peptide pardaxin, which is comparable to that of known native antibacterial peptides.

Table 1

Minimal Inhibitory Concentration (μM) and hemolytic activity of diastereomers pardaxin analogoues.

	Minimal Inhibitory Concentration (μM)						
Peptide	E. coli (D21)	A. calcoaceticus (Ac11)	B. megaterium (Bm11)	M. luteus (ATCC 9341)	S. typhimurium (LT2)	P. aeruginosa (ATCC 27853)	% hemolysis at 50 μM peptide
1.	6	6	0.9	12.5	N.D	N.D	5
2.	12.5	12.5	2.5	N.D[a]	N.D	N.D	0
3.	130	>130	30	N.D	>130	>130	0
4.	7.5	7.5	1.5	N.D	N.D	N.D	0
5.	3.5	3.5	0.75	N.D	N.D	N.D	0
6.	15	6	6	N.D	120	60	0
7.	>120	>120	30	N.D	>120	>120	0
8.	3	3	0.8	5	15	8	100
9.	3	N.D	1.5	N.D	N.D	N.D	83
10.	3	N.D	1.3	N.D	N.D	N.D	56
11.	3	N.D	1.5	N.D	N.D	N.D	82

12.	10	5	1.2	5	N.D	N.D	49	
13.	30	15	3.5	N.D	>100	>100	23	
14.	3.5	1.5	0.6	2.5	N.D	N.D	100	
15.	15	3.5	1.7	N.D	60	60	44	
16.	100	100	50	N.D	>100	>100	0	
17.	10	N.D	1	N.D	N.D	N.D	17	

a-N.D, not determined.

Table 2

Minimum Inhibitory concentration (μM)[a] of the peptides.

Bacterial species	Strain	**8**	**12**	**14**	**1**	Melittin	DermaseptinS	Tetracycline
Escherichia coli	D21	3	10	3.5	6	5	6	1.5
Acinetobacter calcoacetcus	Ac11	3	5	2.5	6	2	3	1.5
Bacillus megaterium	Bm11	0.8	1.2	0.6	0.9	0.3	0.5	1.2
Bacillus subtilis	ATCC-6051	1.5	2	1.5	3	0.6	4	6.5

a. Results are the mean of 3 independent experiments each performed in duplicates, with standard deviation of 20%.

1.4 Membrane destabilizati n induced by the pardaxin-derived peptides. A common property of all of the α-helical, positively charged, naturally-occurring antibacterial peptides studied so far, is their ability to interact and permeate negatively charged phospholipids better than zwitterionic phospholipids. The relevance of these findings to their biological target membranes has been attributed to the fact that the surface of bacteria contains lipopolysaccharides (LPS, in Gram-negative bacteria), and polysaccharides (teichoic acids, in Gram-positive bacteria), both of which are acidic, while normal mammalian cells (e.g., erythrocytes) express the predominantly zwitterionic phospholipid PC on their outer leaflet. The dissipation of the diffusion potential to assess the membrane permeating activity of the peptides on both PC and PC/PS phospholipid vesicles (prepared according to Experimental Procedures, section v) was assayed as described in Experimental Procedures, section xi.. The results shown in Fig. 3 for peptides **1, 8, 12, 14,** indicate that D-amino acids introduced into pardaxin did not significantly affect the ability of the peptides to permeate phospholipid membranes. However, peptide **1,** the only diastereomer that is devoid of hemolytic activity but retains antibacterial activity, permeates negatively charged phospholipids significantly better than zwitterionic phospholipids. As such it behaves similar to native antibacterial peptides, although it is devoid of α-helical structure. The lack of significant intermediate activities with peptides **12** and **14** might be explained by the fact that they both have either the hydrophobic N-helix or the amphipathic C-helix intact, which is sufficient to promote strong binding to both types of vesicles via hydrophobic interactions.

1.5 Visualization of bacterial lysis using electron microscopy. The effect of the pardaxin-derived peptides on the morphology of intact and treated bacteria was visualized using negative staining electron microscopy, as described in Experimental Procedures, section xx. The peptides were added to bacteria at or below their MIC concentration under the same conditions used in the antibacterial assay (see example 1.3 above). Samples were pulled out after an 18 h incubation and were immediately fixed and examined by transmission electron microscopy. Fig. 4 shows the photographs obtained with the non-hemolytic analogue **1,** [D]$P^{7}L^{18}L^{19}$-TApar, as an example. It was found that at the MIC peptide **1** lysed the bacteria completely, and only small fragments could be observed (Fig. 4C). However, at concentrations lower than the MIC, patches were observed on the

bacterial wall (Fig. 4B). These patches might indicate the initial step involved in the lytic process.

EXAMPLE 2. Synthesis and biological activity of melittin-derived diastereomers

2.1 Synthesis. In order to further examine the role of the α-helical structure of cytolysins in their cytotoxicity against mammalian cells and bacteria and to gain insight into the mechanism underlying this effect, four diastereomers of melittin (mel) were synthesized.

Melittin is a 26-mer peptide of the sequence:

Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-
Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2

Modification of the melittin molecule in order to introduce a positive charge was made by converting the acidic C-terminus of melittin or of a fragment thereof to a positive one by reaction of the carboxyl group at the C-terminus with ethylene diamine, in melittin diastereomers in which the N-helix and the C-helix were altered by replacing the two Val residues at positions 5 and 8 of melittin, the Ile residue at position 17 and the Lys residue at position 21 by D-Val, D-Ile and D-Lys, respectively (herein [D]-$V^5V^8I^{17}K^{21}$).

The following melittin-derived diastereomers were found to be non-hemolytic and to exhibit selective cytolytic activity (the bold and underlined residues are D-amino acids):

18. [D]-$V^5V^8I^{17}K^{21}$-mel of the sequence:

Gly-Ile-Gly-Ala-**Val**-Leu-Lys-**Val**-Leu-Thr-Thr-Gly-Leu-
Pro-Ala-Leu-**Ile**-Ser-Trp-Ile-**Lys**-Arg-Lys-Arg-Gln-Gln-NH_2

19. [D]-$V^5V^8I^{17}K^{21}$-mel-COOH of the sequence:

Gly-Ile-Gly-Ala-**Val**-Leu-Lys-**Val**-Leu-Thr-Thr-Gly-Leu-
Pro-Ala-Leu-**Ile**-Ser-Trp-Ile-**Lys**-Arg-Lys-Arg-Gln-Gln-COOH

20. [D]-$V^5V^8I^{17}K^{21}$-[1-22]-TAmel of the sequence:

Gly-Ile-Gly-Ala-**Val**-Leu-Lys-**Val**-Leu-Thr-Thr-Gly-Leu-
Pro-Ala-Leu-**Ile**-Ser-Trp-Ile-**Lys**-Arg-NH-CH_2-CH_2-NH_2

21. [D]-$V^5V^8I^{17}K^{21}$-[4-22]-TAmel of the sequence:

Ala-**Val**-Leu-Lys-**Val**-Leu-Thr-Thr-Gly-Leu-

Pro-Ala-Leu-**Ile**-Ser-Trp-Ile--**Lys**-Arg-NH-CH_2-CH_2-NH_2

The peptides **18-21** were then characterized with regard to their structure, biological function and interaction with bacteria and model membranes composed of either zwitterionic or negatively charged phospholipids.

2.2 CD spectroscopy. The extent of the α-helical structure of the peptides **18 and 19** was determined from their CD spectra in 40% TFE, a solvent that strongly promotes α-helical structure. As expected, the α-helical content of the diastereomers was much lower (80% decrease) than that of melittin, as reflected by the minima at 208 and 222 nm (Fig. 5). The α-helix content of melittin was 73% compared to 15% and 7% in its diastereomers, **18** and **19,** respectively.

2.3 Antibacterial and hemolytic activity of the melittin diastereomers 18-21. The hemolytic activity of the peptides **18-21** against hRBC and their potential to inhibit the growth of different species of bacteria were investigated. The antibiotic tetracycline served as a control in the antibacterial assay. A dose response curve for the hemolytic activity of the peptides was obtained (Fig. 6). Table 3 gives the MIC for a representative set of test bacteria. It can be seen that the introduction of D-amino acids into melittin dramatically reduced its hemolytic activity, which paralleled the loss of the α-helical content in the corresponding analogues. Melittin, with the highest α-helical content was the most hemolytic, while up to the maximum concentration tested (50 μM), peptides **18** and **19**, with the lowest α-helical content, were practically devoid of hemolytic activity. However, despite the dramatic decrease in the hemolytic activity of the melittin diastereomers **18** and **19**, they both retained most of the potent antibacterial activity of the parent peptide. Furthermore, the antibacterial activity of peptide **19** was only slightly lower than that of **18**, which indicates that the amide group at the C-terminus of melittin does not contribute significantly to the antibacterial activity. In contrast, it is known that cecropin with a free carboxylic C-terminal has a significant lower antibacterial activity than that of the native cecropin with an amidated C-terminal (Li et al., 1988).

Table 3

Minimal Inhibitory concentration (μM) and Hemolytic activity of diastereomer melittin analogues.

	Minimal Inhibitory Concentration (μM)					
Peptide designation	E. coli (D21)	A. calcoaceticus (Ac11)	B. megaterium (Bm11)	M. luteus (ATCC 9341)	B. subtilis (ATCC 6051)	% hemolysis at 50 μM peptide
Melittin	5	20	0.3	0.4	0.4	100
18	12	12	0.8	25	3.5	0
19	18	18	1.2	50	8	0
20	8	7	0.8	29	N.D	0
21	21	14	1.2	28	N.D	0
Dermaseptin-S	6	3	0.5	N.D	4	9
Tetracycline	1.5	1.5	1.2	N.D	6.5	–

2.4 Electron microscopy study of bacterial lysis . The effect of the peptide **18** on the morphology of intact and treated bacteria was visualized using transmission electron microscopy. As shown in Fig. 7, at the MIC, the peptide **18** caused total lysis of the bacteria (Fig. 7C). However, at concentrations lower then the MIC, patches were observed on the bacterial wall (Fig. 7B). These patches might represent an initial step in the lytic process.

2.5 Mode of interaction with phospholipid membranes. Since the biological activities of the peptides **18** and **19** were similar, only the mode of interaction of peptide **18** with model phospholipid membranes was compared to that of melittin, in order to elucidate the basis of the membrane selectivity observed. For that purpose the ability of the peptides to dissipate the diffusion potential created in both PC and PC/PS vesicles was measured, and the partition coefficients of the peptides with both types of vesicles, and the localization of the peptide when bound to membranes, were determined.

2.5.1 Membrane permeability induced by the peptides. Various concentrations of melittin and peptide **18** were mixed with vesicles that had been pre-treated with the fluorescent dye, diS-C_2-5, and valinomycin. The kinetics of the fluorescence recovery was monitored with time and the maximum level reached as a function of peptide concentration was determined. As shown in Fig. 8, both melittin and peptide **18** had similar membrane permeating activity with PC/PS vesicles, which demonstrated that introduction of D-amino acids into melittin does not affect the ability of the resulting diastereomer to permeate negatively charged phospholipid (PS/PC) membranes. However, while melittin was also highly active with PC vesicles, the diastereomer was totally devoid of membrane permeating activity with PC vesicles (up to the maximal concentration tested).

2.5.2 Binding Studies. The inability of the diastereomer **18** to permeate PC vesicles may be due to its inability to bind to PC, or alternatively, it may bind to PC vesicles, but once bound cannot organize into structures that induce membrane leakage. In order to differentiate between these two possibilities, a binding study was conducted. The single Trp residue at position 19 of peptide **18** was used as an intrinsic fluorescence probe to follow its binding to PC and PC/PS vesicles. A fixed concentration (~ 0.5 μM) of the peptide was titrated with the desired vesicles (PC or PC/PS) and an increase in the

fluorescence intensity was observed if binding occurred. Plotting of the resulting increases in the fluorescence intensities of Trp as a function of lipid:peptide molar ratios yielded conventional binding curves (Fig. 9A). The binding curve of peptide **18** with PC/PS reveals that almost all the peptide molecules bound to the vesicles at a lipid:peptide molar ratio of 100:1. However, with PC vesicles a net increase in the fluorescence of the Trp was not observed even with the maximal lipid:peptide molar ratio tested, which indicated that the peptide does not bind to PC vesicles. Binding isotherms were constructed by plotting X^*_b (the molar ratio of bound peptide per 60% of the total lipid) versus C_f (the equilibrium concentration of the free peptide in the solution) (Figures 5B). The surface partition coefficients were estimated by extrapolating the initial slopes of the curves to C_f values of zero. The estimated surface partition coefficient, Kp*, of peptide **18** was 1.1±0.2 $x10^4$ M^{-1} (obtained from 4 measurements). This value is similar to the value reported for melittin binding to phosphatidylglycerol/phosphatidylcholine (4.5±0.6 $x10^4$ M^{-1}) (Beschiaschvili and Seelig, 1990).

The shape of the binding isotherm of a peptide can provide information on the organization of the peptide within membranes (Schwarz et al., 1987). The binding isotherm of peptide **18** bend downward indicating a negative cooperativity. A possible explanation for this negative cooperativity is that at low concentration, peptide **18** binding to PS/PC is enhanced by the negative charge of the phospholipid headgroups compared to the partition equilibrium with no charge effect. In addition, upon binding to the membrane the peptide partially neutralizes the negative membrane surface charge. However, once the membrane surface charge is neutralized, further peptide **18** binding is difficult, since repulsion of like charges becomes the dominant factor. Similar results were obtained in studies of melittin binding to negatively charged phospholipid membranes) (Batenburg et al., 1987; Beschiaschvili and Seelig, 1990). Interestingly, unlike melittin which binds strongly also to PC vesicles (Kuchinka and Seelig, 1989), peptide **18** did not bind to PC vesicles.

2.6 Tryptophan Quenching Experiments. A tryptophan residue naturally present in the sequence of a protein or a peptide can serve as an intrinsic probe for the localization of the peptide within a membrane. Melittin contains a tryptophan residue at position 19, the N-terminal side of the C-helix. With both melittin and peptide **18**, the largest quenching

of tryptophan fluorescence was observed with 6,7-Br-PC/PS vesicles (Fig. 10). Less quenching was observed with 9,10-Br-PC/PS, and the least with 11,12-Br-PC/PS. These results indicate that upon binding to vesicles, the peptides were located near the head groups of the phospholipids.

EXAMPLE 3. Synthesis and biological activity of model Lys/Leu diastereomers

3.1 Lys/Leu diastereomers design. Six diastereomers of short linear model 12-mer peptides composed of varying ratios of lysine and leucine were synthesized in order (1) to examine whether a balance between hydrophobicity and a net positive charge may be a sufficient criteria necessary for selective bacterial lysis, and (2) to gain insight into the mechanism underlying this effect.

In the first series of model Lys/Leu 12-mer peptides **22-25**, one third of their sequence was composed of D-amino acid residues. The location of the D-amino acids remained constant in all peptides which was constructed for maximum disruption of α-helical structure. D-amino acids were distributed along the peptide, leaving only very short stretches of 1-3 consecutive L-amino acids. The following peptides were synthesized:

22. [D]-$L^{3,4,8,10}$-K_3L_9 of the sequence:

Lys-Leu-**Leu-Leu**-Leu-Leu-Lys-**Leu** -Leu-**Leu**-Leu-Lys-NH_2

23. [D]-$L^{3,4,8,10}$-K_4L_8, of the sequence

Lys-Leu-**Leu-Leu**-Lys-Leu-Leu-**Leu** -Lys-**Leu**-Leu-Lys-NH_2

24. [D]-$L^{3,4,8,10}$-K_5L_7, of the sequence

Lys-Leu-**Leu-Leu**-Lys-Leu-Lys-**Leu** -Lys-**Leu**-Leu-Lys-NH_2

25. [D]-$L^{3,4,8,10}$-K_7L_5 of the sequence :

Lys-Lys-**Leu-Leu**-Lys-Leu-Lys-**Leu** -Lys-**Leu**-Lys-Lys-NH_2

In the second series of model Lys/Leu 12-mer peptides **26-27**, two thirds of their sequence were composed of D-amino acid residues, at the exact positions of the L-amino acid residues of peptides **23** and **24** as follows:

26. [D]-$K^{1,5,9,12}$ $L^{2,6,7,11}$ - K_4L_8, of the sequence:

Lys-Leu-Leu-Leu-**Lys-Leu-Leu**-Leu -**Lys**-Leu-**Leu-Lys**-NH_2

27. [D]-$K^{1,5,7,9,12}$ $L^{2,6,11}$-K_5L_7, of the sequence:

Lys-Leu-Leu-Leu-**Lys-Leu-Lys**-Leu-**Lys**-Leu-**Leu-Lys**-NH_2

In a third series of model Lys/Leu peptides. a 6-mer and a 8-mer diastereomers were synthesized (peptides **28** and **29** , respectively):

28. [D]- $L^{2,4}$-K_2L_4 , of the sequence:

Lys-**Leu**-Leu-**Leu** -Leu-Lys

29. [D]-$L^{2,4,6}$-K_3L_5 , of the sequence:

Lys-**Leu**-Leu-**Leu** - Lys-**Leu**-Leu-Lys

Further Lys/Leu diastereomers according to the invention that were synthesized:

30. Lys **Leu** Leu Leu Lys **Leu** Lys **Leu** Lys Leu **Leu** Lys

31. Lys **Leu Leu Leu** Lys Leu Lys **Leu** Lys **Leu Leu** Lys

32. Lys Leu **Leu** Leu **Lys** Leu Lys **Leu Lys** Leu **Leu** Lys

3.2 Synthesis of Lys/Leu diastereomers - The peptides were synthesized as described in Experimental Procedures, section (ii). The peptides were then characterized with regard to their structure, biological function and interaction with bacteria and model membranes composed of either zwitterionic or negatively charged phospholipids.

3.3 Hydrophobicity. The hydrophobicities and net positive charges of the peptides **22-25** are listed in Table 4. Mean values of hydrophobicity were calculated using consensus value of hydrophobicity scale (Eisenberg et al., 1984). As shown in Fig. 11, a direct correlation was found between hydrophobicity and the retention time of the peptides, suggesting that structure does not significantly contribute to overall hydrophobic interactions with the stationary phase.

Table 4

Hydrophobicity and net charge of the Leu/Lys diastereomers.

Peptide Designation	Net Charge	Hydrophobicity
22.	+4	0.12
23.	+5	- 0.01
24.	+6	- 0.15
25.	+8	- 0.42

3.4 CD spectroscopy. The extent of the α-helical structure of the diastereomers **22-25** was determined from their CD spectra in 40% TFE. As expected, after incorporation of D-amino acids, no signal was observed for all the diastereomers, demonstrating the lack of any specific secondary structure (data not shown). It is to be noted that in a recent study, a peptide with a sequence identical to that of peptide **23**, but composed of only L-amino acids, was found to have about 40% α-helical structure in methanol and in DMPC vesicles (Cornut et al., 1994).

3.5 Antibacterial and hemolytic activity of the peptides 22-29. The hemolytic activity of the peptides **22-29** against hRBC was tested. A dose response curve for the hemolytic activity of the peptides **22-25** is shown in Fig. 12 wherein the hemolytic activity of melittin served as a control. A direct correlation was found between the hydrophobicity (Table 4) and the hemolytic activity of the diastereomers. Peptide **22,** [D]-$L^{3,4,8,10}$-K_3L_9, which has the highest hydrophobicity, was the most hemolytic peptide. However, its hemolytic activity is very low in comparison to melittin (>60 fold less activity). All the other peptides showed no significant hemolytic activity up to the maximum concentration tested (100 μM). The hemolytic activity of peptides **22-29** is shown in Table 5. It should be noted that although peptide **23**, [D]-$L^{3,4,8,10}$-K_4L_8, is not hemolytic at concentrations >100 fold of those required for significant hemolysis by melittin, its entirely L-amino acid

form has been shown in a recent study to have hemolytic activity similar to that of melittin (~5 fold less) (Cornut et al., 1994).

The peptides **22-29** were also tested for their antibacterial activity against a representative set of bacteria, in which tetracycline, dermaseptin S, and melittin served as controls. The resultant MICs are shown in Table 5. The data show that the antibacterial activity of the diastereomers **22-29** was modulated by the balance between hydrophobicity and positively charged amino acids. Both the most hydrophobic peptide **22** and the most hydrophilic peptide **25** displayed the lowest range in antibacterial activity (Table 5). However, peptides **23** and **24** displayed high antibacterial activity against most of the bacteria tested with the former being slightly more potent. Furthermore, each peptide had a unique spectrum of antibacterial activity, and each was active more against Gram-positive as compared to Gram-negative bacteria.

3.6 Synergistic effects between tetracycline and the Lys/Leu diastereomers in serum. To investigate a possible synergistic relationship between the antibiotic tetracycline and the diastereomers, tetracycline was tested in 2-fold serial dilutions against *Pseudomonas aeruginosa* (ATCC 27853) in the presence of a constant equimolar concentration (1 μM) of peptide **24,** [D]-$L^{3,4,8,10}$-K_5L_7. Antibacterial activity of the mixtures was determined as described in Experimental procedures, section (xii).

A synergistic effect was observed between tetracycline and the diastereomer **24.** Tetracycline shows little activity against *P. aeruginosa*. However, when mixed with 1μM solution of peptide **24**, a concentration which is 10 fold lower than that required for lytic activity against *P. aeruginosa*, an eight fold increase in the activity of tetracycline was observed (Table 6). A possible explanation for the synergistic effect is that the peptide slightly disrupts the bacterial wall which improves partitioning of tetracycline into the bacteria. This is supported by electron microscopy studies which show that below its MIC, peptide **24** causes morphological changes in the bacterial wall (Fig. 14). In addition, the effect of pooled human serum on the antibacterial activity of peptide **24** and the native antibacterial peptide dermaseptin against *P. aeruginosa* and *E. coli* was found to differ considerably (Table 6). While dermaseptin was 8-10 fold less active in the presence of serum, peptide **24** retained its antibacterial activity.

Table 5

Minimal Inhibitory Concentration (μM) of the peptides.

	Minimal Inhibitory Concentration [a] (μM)					
Peptide Designation	*E. Coli*	*A. calcoaceticus*	*P. aeruginosa*	*B. megaterium*	*B. subtilis*	% Hemolysis
	(D21)	(Ac11)	(ATCC-27853)	(Bm11)	(ATCC-6051)	at 100 μM
22	9	20	125	0.7	1.1	58
23	3.5	4	10	0.4	0.5	0
24	7	20	10	0.25	2	0
25	80	200	>200	1	100	0
26	4	N.D	N.D	0.5	N.D	0
27	7	N.D	N.D	0.2	N.D	0
28	200	N.D	N.D	50	N.D	0
29	3	N.D	N.D	3	N.D	0
Dermaseptin S	6	3	25	0.5	4	–
Melittin	5	2	25	0.3	0.6	–
Tetracycline	1.5	1.5	50	1.2	6.5	–

a. Results are the mean of 3 independent experiments each performed in duplicates, with standard deviation of 20%

Table 6

Minimal Inhibitory Concentration (μM)[a] in the presence of human serum and synergistic activity of peptide **24**

	Minimal Inhibitory Concentration (μM)			
	P. aeruginosa (ATCC-27853)		*E. coli* (D21)	
Peptide Designation	0% Serum	33% Serum	0% Serum	33% Serum
24	10	10	7	7
Dermaseptin S	25	200	6	50
Tc[b]	50			
Tc+**24** (1 μM)	6			

[a]. Results are the mean of 2 independent experiments each performed in duplicates, with standard deviation of 20%.

[b]. Tc - Tetracycline

3.7 Peptide-induced membrane permeation. Various concentrations of peptides were mixed with vesicles that had been pretreated with the fluorescent dye, diS-C_2-5, and valinomycin. The kinetics of the fluorescence recovery was monitored and the maximum fluorescence level was determined as a function of peptide concentration (Fig. 13). PC/cholesterol vesicles (10:1) served as a model of the phospholipid composition of the outer erythrocyte leaflet (Verkleij et al., 1973), and PE/PG vesicles (7:3) was used to mimic the phospholipid composition of *E. coli* (Shaw, 1974). A direct correlation was found between the potential of the peptides to permeate model phospholipid membranes and their lytic activity against erythrocytes and *E. coli* . Only the hemolytic peptide **22** permeated the zwitterionic phospholipid vesicles. Furthermore, the ability of the peptides to permeate PE/PG vesicles correlates with the antibacterial activity of the peptides against *E. coli* (Table 5). Peptide **25**, which has the lowest antibacterial activity, also had significantly decreased ability to permeate PE/PG vesicles compared to the other three peptides **22-24.**

3.8 Electron microscopy study of bacterial lysis. The effect of the diastereomers **22-25** on the morphology of treated *E. coli* was visualized using transmission electron microscopy. All the peptides caused total lysis of the bacteria at the MIC (data not shown). However, when the peptides were utilized at concentrations corresponding to 80% of their MIC, some differences in the morphology of the treated bacteria were observed, depending upon the peptide used. The most hydrophobic peptide **22** caused the most damage to the cell wall and membranes, while the least hydrophobic peptide **25** only caused local perturbations (Fig. 14).

EXAMPLE 4. Synthesis and biological activity of model Lys/Ala and Lys/Val diastereomers.

4.1 Diastereomer design. To further examine whether modulating hydrophobicity and the net positive charge of linear cytotoxic peptides is sufficient to confer selective antibacterial activity, two further model 12-mer peptides **33** and **34-37**, composed of Lys/ Ala or Lys/Val residues, respectively, with at least one third of their sequences being of D-Ala or D-Val residues, were synthesized:

33. [D]-$A^{3,4,8,10}$-K_4A_8 of the sequence:

Lys-Ala-**Ala-Ala-**Lys-Ala-Ala-**Ala-**Lys-**Ala**-Ala-Lys-NH_2

34. [D]-$V^{3,4,8,10}$-K_4V_8 of the sequence:

Lys-Val-**Val-Val-**Lys-Val-Val-**Val-**Lys-**Val**-Val-Lys-NH_2

35. Lys **Val** Val Val Lys **Val** Lys **Val** Lys Val **Val** Lys

36. **Lys Val Val Val** Lys Val Lys **Val** Lys **Val Val** Lys

37. Lys Val **Val** Val **Lys** Val Lys **Val Lys** Val **Val** Lys

4.2 Synthesis. The Lys/Ala and Lys/Val diastereomers were synthesized as described in Experimental Procedures, section (ii).

4.3 Antibacterial and hemolytic activity. Peptides **33** and **34** were tested against *E. coli* and *B. megaterium* and hRBC. The results in Table 7 show that both model diastereomers are antibacterial and non-hemolytic:

Table 7

Minimal Inhibitory Concentration (μM) and hemolytic activity of the peptides 28 and 29

Peptide Designation	Minimal Inhibitory Concentration (μM) *E. coli* (D21)	*B. megaterium* (Bm11)	% hemolysis at 100 μM
33	12	1	0
34	3.5	0.8	0

EXAMPLE 5. Synthesis of further model diastereomers

The following model diastereomers according to the invention composed of sequences of 6, 8, 12, 14, 16, 19, 25, 26 and 30 residues of two, three or more different amino acids, were synthesized:

38. Lys **Leu** Ile Leu Lys **Leu**

39. Lys **Val** Leu His Leu **Leu**

40. Leu **Lys** Leu Arg Leu **Leu**

41. Lys Pro Leu **His** Leu Leu

42. Lys **Leu Ile Leu** Lys **Leu** Val Arg

43. Lys **Val** Phe **His Leu Leu** His Leu

44. **His Lys** Phe **Arg** Ile **Leu Lys** Leu

45. Lys Pro Phe His **Ile** Leu His Leu

46. **Lys Ile** Ile Ile Lys **Ile** Lys **Ile** Lys Ile **Ile** Lys

47. Lys **Ile Ile Ile** Lys Ile Lys **Ile** Lys **Ile Ile** Lys

48. **Lys** Ile **Ile** Ile **Lys** Ile Lys **Ile Lys** Ile **Ile** Lys

49. Lys Ile Pro Ile Lys Ile **Lys** Ile Lys Ile Pro Lys

50. Lys Ile Pro Ile Lys **Ile** Lys Ile Lys **Ile** Val Lys

51. Arg **Ile** Ile **Ile** Arg **Ile** Arg **Ile** Arg Ile **Ile** Arg

52. Arg **Ile Ile Ile** Arg **Ile Arg Ile** Arg **Ile Ile** Arg

53. **Arg** Ile **Ile** Ile **Arg** Ile Arg **Ile Arg** Ile **Ile** Arg

54. Arg **Ile** Val Ile Arg **Ile** Arg **Ile** Arg Leu **Ile** Arg

55. Arg Ile Ile Val Arg Ile Arg Leu Arg Ile Ile Arg

56. Arg Ile Gly Ile Arg Leu Arg Val Arg Ile Ile Arg

57. Lys Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg

58. Arg Ile Ala Val Lys Trp Arg Leu Arg Phe Ile Lys

59. Lys Ile Gly Trp Lys Leu Arg Val Arg Ile Ile Arg

60. Lys Lys Ile Gly Trp Leu Ile Ile Arg Val Arg Arg

61. Arg Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg Ile Arg

62. Arg Ile Ile Val Arg Ile Arg Leu Arg Ile Ile Arg Val Arg

63. Arg Ile Gly Ile Arg Leu Arg Val Arg Ile Ile Arg Arg Val

64. Lys Ile Val Ile Arg Ile Arg Ala Arg Leu Ile Arg Ile Arg Ile Arg

65. Arg Ile Ile Val Lys Ile Arg Leu Arg Ile Ile Lys Lys Ile Arg Leu

66. Lys Ile Gly Ile Lys Ala Arg Val Arg Ile Ile Arg Val Lys Ile Ile

67. Arg Ile Ile Val His Ile Arg Leu Arg Ile Ile His His Ile Arg Leu

68. His Ile Gly Ile Lys Ala His Val Arg Ile Ile Arg Val His Ile Ile

69. Arg Ile Tyr Val Lys Ile His Leu Arg Tyr Ile Lys Lys Ile Arg Leu

70. Lys Ile Gly His Lys Ala Arg Val His Ile Ile Arg Tyr Lys Ile Ile

71. Arg Ile Tyr Val Lys Pro His Pro Arg Tyr Ile Lys Lys Ile Arg Leu

72. Lys Pro Gly His Lys Ala Arg Pro His Ile Ile Arg Tyr Lys Ile Ile

73. Lys Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg Ile Arg Ile Arg Lys Ile Val

74. Arg Ile Ile Val Lys Ile Arg Leu Arg Ile Ile Lys Lys Ile Arg Leu Ile Lys Lys

75. Lys Ile Gly Trp Lys Leu Arg Val Arg Ile Ile Arg Val Lys Ile Gly Arg Leu Arg

76. Lys Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg Ile Arg Ile Arg Lys Ile Val Lys Val Lys Arg Ile Arg

77. Arg Phe Ala Val Lys Ile Arg Leu Arg Ile Ile Lys Lys Ile Arg Leu Ile Lys Lys Ile Arg Lys Arg Val Ile Lys

78. Lys Ala Gly Trp Lys Leu Arg Val Arg Ile Ile Arg Val Lys Ile Gly Arg Leu Arg Lys Ile Gly Trp Lys Lys Arg Val Arg Ile Lys

79. Arg Ile Tyr Val Lys Pro His Pro Arg Tyr Ile Lys Lys Ile Arg Leu

80. Lys Pro Gly His Lys Ala Arg Pro His Ile Ile Arg Tyr Lys Ile Ile

81. Lys Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg Ile Arg Ile Arg Lys Ile Val

82. Arg Ile Ile Val Lys Ile Arg Leu Arg Ile Ile Lys Lys Ile Arg Leu Ile Lys Lys

83. Arg **Ile Tyr Val Ser** Lys **Ile Ser** Ile **Tyr Ile** Lys **Lys Ile** Arg **Leu**

84. Lys **Ile** Val **Ile Phe Thr Arg Ile** Arg **Leu Thr Ser Ile** Arg **Ile Arg** Ser **Ile** Val

85. **Lys Pro Ile** His **Lys** Ala **Arg Pro Thr Ile Ile** Arg **Tyr Lys** Met **Ile**

EXAMPLE 6. Synthesis and biological activity of cyclic diastereomers.

6.1 Design. The following cyclic derivatives of diastereomers of pardaxin fragments with cysteine residues at both the N- and C-termini were synthesized:

86. Cyclic K^1[D]P^7 $L^{18}L^{19}$ [1-22]-par of the sequence:

Cys-Lys-Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-Ser-
Ser-Pro-Leu-Phe-Lys-Thr-**Leu-Leu**-Ser-Ala-Val-Cys

87. Cyclic K^1 K^2[D]P^7 $L^{18}L^{19}$ [1-22]-par of the sequence:

Cys-Lys-Lys-Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-Ser-
Ser-Pro-Leu-Phe-Lys-Thr-**Leu-Leu**-Ser-Ala-Val-Cys

88. Cyclic K^1 K^2K^3 [D]P^7 $L^{18}L^{19}$ [1-22]-par of the sequence:

Cys-Lys-Lys-Lys-Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-
Ser-Ser-Pro-Leu-Phe-Lys-Thr-**Leu-Leu**-Ser-Ala-Val-Cys

The following cyclic derivatives of diastereomers of different amino acid residues with cysteine residues at both the N- and C-termini were synthesized:

89. Cyclic Cys Arg **Ile** Val Ile Arg **Ile** Arg **Ile** Arg Leu **Ile** Arg **Ile** Arg Cys

90. Cyclic Cys **Lys Pro Gly** His **Lys** Ala **Arg Pro His Ile Ile** Arg **Tyr Lys** Ile **Ile** Cys

91. Cyclic Cys Arg **Phe Ala Val** Lys **Ile Arg Leu** Arg **Ile Ile** Lys **Lys Ile** Arg **Leu Ile** Lys **Lys Ile Arg Lys** Arg **Val Ile** Lys Cys

92. Cyclic Cys Lys Leu **Leu Leu** Lys Leu Leu **Leu** Lys **Leu** Leu Lys Cys

93. Cyclic Cys Lys Leu **Leu Leu** Lys Leu Lys **Leu** Lys **Leu** Leu Lys Cys

The following cyclic derivatives of diastereomers of different amino acid residues without cysteine residues at both the N- and C-termini were synthesized:

94. HN - Lys Leu **Leu** **Leu** Lys Leu Leu **Leu** Lys **Leu** Leu Lys – CO

95. HN - Lys Leu **Leu** **Leu** Lys Leu Lys **Leu** Lys **Leu** Leu Lys – CO

6.2 Synthesis of the cyclic diastereomers. The cyclic peptides were synthesized by a solid-phase method as described in Experimental Procedures, section (ii), without or with cysteine residues at both the N and C-termini of the peptides. The cyclization without cystein was carried out by protecting the N-terminal, activating the C-terminal, deprotection of the N-terminal and reaction of the C- and N-terminal groups while still bound to the resin. After HF cleavage and RP-HPLC purification the peptides were solubilized at low concentration in PBS (pH 7.3), and cyclization was completed after 12 h. The cyclic peptides were further purified on RP-HPLC and subjected to amino acid analysis to confirm their composition, and SDS-PAGE to confirm their monomeric state.

6.3 Antibacterial and hemolytic activity. Peptides **86-88** were tested against *E. coli* and *B. megaterium* and hRBC. The results in Table 8 show that all three cyclic pardaxin-derived diastereomers are antibacterial and non-hemolytic:

Table 8

Minimal Inhibitory Concentration (μM) and hemolytic activity of the cyclic pardaxin-derived diastereomers.

Peptide Designation	Minimal Inhibitory Concentration (μM)		
	E. coli (D21)	*B. megaterium* (Bm11)	% hemolysis at 100 μM
86	30	10	0
87	15	6	0
88	7.5	2	0

6.4 Antibacterial and hemolytic activity. Peptides **92-95** were tested against *E. coli, B. subtilis* and *P aeruginosa.* The results in Table 8a show that all four cyclic diastereomers are antibacterial and non-hemolytic:

Table 8a

Minimal Inhibitory Concentration (μM) and hemolytic activity of the cyclic diastereomers.

	Minimal Inhibitory Concentration (μM)			
Peptide Designation	*E. coli*	*B. subtilis*	*P. aeruginosa*	% hemolysis at 50 μM
92	12.5	1.2	25	0
93	15	5	25	0
94	12.5	1.5	30	0
95	15	6	20	0

EXAMPLE 7. Synthesis and biological activity of bundled Lys/Leu peptide diastereomers.

7.1 Design. Using as template peptide **23** and as monomers peptide **23** or **24** with an additional cysteine residue at the C-terminus (**23C** and **24C**, respectively, the following bundle-sequences were produced:

96. ([D]-$L^{3,4,8,10}$-$K_4L_8C)_5$ [D]-$L^{3,4,8,10}$-K_4L_8 of the sequence:

(Lys-Leu-**Leu-Leu**-Lys-Leu-Leu-**Leu**-Lys-**Leu**-Leu-Lys-Cys-$NH_2)_5$ Lys-Leu-**Leu-Leu**-Lys-Leu-Leu-**Leu** -Lys-**Leu**-Leu-Lys-NH_2

97. ([D]-$L^{3,4,8,10}$-$K_5L_7C)_5$ [D]-$L^{3,4,8,10}$-K_4L_9 of the sequence:

(Lys-Leu-**Leu-Leu**-Lys-Leu-Lys-**Leu**-Lys-**Leu**-Leu-Lys-Cys-$NH_2)_5$ Lys-Leu-**Leu-Leu**-Lys-Leu-Leu-**Leu** -Lys-**Leu**-Leu-Lys-NH_2

7.2 Synthesis. In order to produce template-bound diastereomers, 1:1 molar ratio of DCC and bromoacetic acid were allowed to react in DMSO at 25 °C for 1h. The template

(peptide **23**) was added to the reaction mixture and left under agitation for 12 h after which the DMSO was lyophilized. The remaining bromoacetic acid was extracted with dry ether. The template was then reacted with excess of diastereomers **23C** and **24C** with cysteine residue at their C-terminus, in PBS pH 7.3 at 25 °C for 1 h. The template-bound diastereomers **96** and **97** were further purified on RP-HPLC, and examined on SDS-PAGE to confirm their aggregation state.

7.3 Antibacterial and hemolytic activity. The template-bound diastereomers diastereomers **96** and **97** were tested against *E. coli* and *B. megaterium* and hRBC. The results in Table 9 show that both bundle sequences are antibacterial and non-hemolytic.

Table 9

Minimal Inhibitory Concentration (μM) and hemolytic activity of the bundles.

	Minimal Inhibitory Concentration (μM)		
Peptide Designation	*E. coli* (D21)	*B. megaterium* (Bm11)	% hemolysis at 100 μM
96	0.2	0.05	0
97	0.1	0.02	0

EXAMPLE 8. Synthesis and biological activity of mixtures of Lys/Leu 12-mer peptide diastereomers. Peptides were synthesized by a solid phase method as described in Experimental Procedures, section (ii) above. At each coupling step a mixture composed of 1 eq each of lysine, leucine and D-leucine was added to the reaction. The synthesis resulted in a mixture of 3^{12} different peptides. After HF cleavage the peptides were extracted with double distilled water (ddw) and lyophilized.

The mixture of the Lys/Leu 12-mer peptide diastereomers was tested against *E. coli* D21 (MIC: 15 μg/ml) and *B. megaterium* Bm11 D21 (MIC: 3 μg/ml) and hRBC (0% hemolysis at 100 μM). As expected, the mixture had antibacterial activity but was non-hemolytic.

EXAMPLE 9. Synthesis and biological activity of Lys/Leu/D-Leu random copolymers. In order to produce diastereomers of polymers of different sizes, excess of N-carboxyanhydride residues over initiator free amino acids were allowed to polymerize in DMF at 25 °C for 4 h (Katchalski and Sela, 1958). Polymers consisting of different ratios of lysine, leucine and D-leucine were produced using different ratios of lysine-N-carboxyanhydride, leucine-N-carboxyanhydride and D-leucine-N-carboxy anhydride. Three of such polymers and their antibacterial and hemolytic activity are shown in Table 10.

Table 10

Minimal Inhibitory Concentration (μM) and hemolytic activity of the Lys/Leu/D-Leu copolymers.

Amino Acids Ratio (Molar) Lys: Leu: [D]-Leu	Minimal Inhibitory Concentration (μg/ml) *E. coli* (D21)	*megaterium* (Bm11)	% hemolysis at 100 μM
1 : 1 : 1	90	15	0
2 : 1 : 1	35	8	0
3 : 1 : 1	80	20	0

EXAMPLE 10. Antifungal activity of the diastereomers - The antifungal activity of the pardaxin-derived peptides **1** and **16** (see Example 1 above) was examined in sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 100 μL as follows: Fifty microliters of a suspension containing fungi at concentration of 1 X 10^6 Colony-Forming Units (CFU)/ml in culture medium (Sabouraud's glucose broth medium) was added to 50 μL of water containing the peptide in serial 2-fold dilutions in water. Inhibition of growth was determined by measuring the absorbance at 492 nm with a Microplate autoreader El309 (Bio-tek Instruments), after an incubation time of 48 h at 30°C. Antifungal activities were expressed as the minimal inhibitory concentration (MIC), the concentration at which

100% inhibition of growth was observed after 48 h of incubation. The fungi used were: *Candida albicans* (IP886-65) and *Cryptococcus neoformans* (IP960-67). As shown in Table 11, both peptides **1** and **16** showed antifungal activity.

Table 11

Minimal Inhibitory Concentration (μM) of the diastereomers **1** and **16** against fungi.

	Minimal Inhibitory Concentration (μM)	
Peptide Designation	*Candida albicans (IP886-65)*	*Cryptococcusneoformans (IP960-67)*
1	35	50
16	120	150

EXAMPLE 11. Anticancer activity of the diastereomers. The anticancer activity of the Lys/Leu diastereomers **23** and **24** (see Example 3 above) was examined against mouse adenocarcinoma. Cells were seeded at 5-10 000/well in 96-well microtiter plates in Dulbecco's modified Eagle's medium. After the cells had attached, 20 μl of diluted peptide solution in normal saline were transferred to the well to give final concentrations ranging from 20 to 150 μM. Following 1h incubation with the peptides, the viability of the cancer cell was measured by Trypan blue (0.1% w/v) vital staining assay. In control experiments the peptide solvent alone was added to the cells. Anticancer activities were expressed as the minimal inhibitory concentration (MIC), the concentration at which 100% inhibition of growth was observed after 1 h of incubation. The results in Table 12 show that both peptides are active against malignant cells.

Table 12

Minimal Inhibitory Concentration (μM) of the diastereomers against mouse adenocarcinoma.

	Minimal Inhibitory Concentration (μM)
Peptide Designation	mouse adenocarcinoma
23	50
24	80

EXAMPLE 12. Activity of the diastereomers against Leishmania mexicana. The melittin-derived diastereomer peptide **20** (see Example 2 above) and the Leu/Lys diastereomer peptide **23** (see Example 3 above) were tested against *Leishmania.* Promastigotes of the *Leishmania mexicana* NR strain to be assayed were cultured at 27 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum. Parasite were harvested by centrifugation at 1200 x g for 10 min at 4 °C and washed twice with PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7). The washed promastigotes were counted in a hemocytometer and adjusted to $1x\ 10^6$ parasites/ml. Aliquotes of this suspension were assayed in a final volume of 100 μl by counting living (motile) cells after 24 h of incubation at 26 °C in the absence or presence of various concentrations of the diastereomers. Anti -*Leishmania* activities were expressed as the minimal inhibitory concentration (MIC), the concentration at which 100% death was observed after 24 h of incubation. It was found that for peptide **23** the MIC is 17 μM and for peptide **20** the MIC is 32 μM.

EXAMPLE 13. Antiviral activity of the diastereomer 23 - Sendai virus (Z strain) was grown in the allantoic sac of 10-11 day old embryonated chicken eggs, harvested 48 h after injection and purified. The virus was resuspended in buffer composed of 160 mM NaCl, 20 mM tricine, pH 7.4, and stored at -70 °C. Virus haemagglutinating activity was measured in haemagglutinating units (HAU). One microlitre contained ~ 60000 HAU.

Fresh human blood was obtained from a blood bank and stored for up to 1 month at 4 °C. Prior to use, erythrocytes were washed twice with PBS pH 7.2, and diluted to the desired concentration (% v/v) with the same buffer. Virions, erythrocytes and peptides were mixed in different orders of addition and various amounts. The final incubation was always at 37 °C for 60 min, followed by centrifugation at 5700 g for 10 min to remove intact cells. In all cases duplicate samples were used and two aliquots were taken from the supernatant of each sample to two wells of a 96-well plate. The amount of hemoglobin release was monitored by measuring the absorbance of the wells with the ELISA plate reader at 540 nm. Antiviral activity was expressed as the minimal inhibitory concentration (MIC), the concentration at which no release of hemoglobin was observed after incubation. It was found that for the Lys/Leu diastereomer peptide **23** the MIC is 80 μM.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(ii) TITLE OF INVENTION: ANTIPATHOGENIC SYNTHETIC PEPTIDES AND COMPOSITIONS COMPRISING THEM

(iii) NUMBER OF SEQUENCES: 95

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER:
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/IL97/00066
(B) FILING DATE: 20-FEB-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 1

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(7, 18, 19)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:33
(D) OTHER INFORMATION:/product= "OTHER"
/note= "two aminoethylamino groups are attached at the C-terminus"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Gly Gln
20 25 30

Glu

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 2

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(7, 18, 19)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:22
(D) OTHER INFORMATION:/product= "OTHER"
/note= "an aminoethylamino group is attached at the C-terminus"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val
20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 3

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(7, 18, 19)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val
20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 4

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(8, 19, 20)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:23
(D) OTHER INFORMATION:/product= "OTHER"
/note= "an aminoethylamino group is attached at the C-terminus"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Lys Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe
1 5 10 15

Lys Thr Leu Leu Ser Ala Val
20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 5

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(9, 20, 21)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:24
(D) OTHER INFORMATION:/product= "OTHER"
/note= "an aminoethylamino group is attached at the C-terminus"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Lys Lys Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu
1 5 10 15

Phe Lys Thr Leu Leu Ser Ala Val
20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 6

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(9, 20, 21)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Lys Lys Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu
1 5 10 15

Phe Lys Thr Leu Leu Ser Ala Val
20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 7

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:7
(D) OTHER INFORMATION:/product= "D-AMINO ACID RESIDUE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:11
(D) OTHER INFORMATION:/product= "OTHER"
/note= "AN AMINOETHYLAMINO GROUP IS ATTACHED AT THE C-TERMINUS"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 8

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:33
(D) OTHER INFORMATION:/product= "OTHER"
/note= "TWO AMINOETHYLAMINO GROUPS ARE ATTACHED AT THE C-TERMINUS"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Gly Gln
20 25 30

Glu

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 9

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:13
(D) OTHER INFORMATION:/product= "D-AMINO ACID RESIDUE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:33
(D) OTHER INFORMATION:/product= "OTHER"
/note= "TWO AMINOETHYLAMINO GROUPS ARE ATTACHED AT THE C-TERMINUS"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Gly Gln
20 25 30

Glu

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 10

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(5, 19)
(D) OTHER INFORMATION:/product= "D-AMINO ACID RESIDUES"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:33
(D) OTHER INFORMATION:/product= "OTHER"
/note= "TWO AMINOETHYLAMINO GROUPS ARE ATTACHED AT THE C-TERMINUS"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Gly Gln
20 25 30

Glu

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: linear

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: peptide 11

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(7, 19)
(D) OTHER INFORMATION:/product= "D-AMINO ACID RESIDUES"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:33
(D) OTHER INFORMATION:/product= "OTHER"
/note= "TWO AMINOETHYLAMINO GROUPS ARE ATTACHED AT THE C-TERMINUS"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Gly Gln
20 25 30

Glu

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 12

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:7
(D) OTHER INFORMATION:/product= "D-AMINO ACID RESIDUE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:33
(D) OTHER INFORMATION:/product= "OTHER"
/note= "TWO AMINOETHYLAMINO GROUPS ARE ATTACHED AT THE C-TERMINUS"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Gly Gln
20 25 30

Glu

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 13

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:7
(D) OTHER INFORMATION:/product= "D-AMINO ACID RESIDUE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Gly Gln
20 25 30

Glu

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 14

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(18, 19)
(D) OTHER INFORMATION:/product= "D-AMINO ACID RESIDUES"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:33
(D) OTHER INFORMATION:/product= "OTHER"
/note= "TWO AMINOETHYLAMINO GROUPS ARE ATTACHED TO THE C-TERMINUS"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Gly Gln
20 25 30

Glu

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 15

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(18, 19)
(D) OTHER INFORMATION:/product= "D-AMINO ACID RESIDUES"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Gly Gln
20 25 30

Glu

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 16

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(7, 18, 19)
(D) OTHER INFORMATION:/product= "D-AMINO ACID RESIDUES"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Gly Gln
20 25 30

Glu

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 17

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:7
(D) OTHER INFORMATION:/product= "D-AMINO ACID RESIDUE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:22
(D) OTHER INFORMATION:/product= "OTHER"
/note= "AN AMINOETHYLAMINO GROUP IS ATTACHED AT THE C-TERMINUS"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val
20

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 18

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(5, 8, 17, 21)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:26
(D) OTHER INFORMATION:/product= "OTHER"
/note= "the carboxyl group at the C-terminus is replaced by an
amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro Ala Leu
1 5 10 15

Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln
20 25

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 19

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(5, 8, 17, 21)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro Ala Leu
1 5 10 15

Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln
20 25

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 20

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(5, 8, 17, 21)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:22
(D) OTHER INFORMATION:/product= "OTHER"
/note= "an aminoethylamino group is attached at the C-terminus"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro Ala Leu
1 5 10 15

Ile Ser Trp Ile Lys Arg
20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 21

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 5, 14, 18)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:19
(D) OTHER INFORMATION:/product= "OTHER"
/note= "an aminoethylamino group is attached at the C-terminus"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro Ala Leu Ile Ser Trp
1 5 10 15

Ile Lys Arg

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 22

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 4, 8, 10)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:12
(D) OTHER INFORMATION:/product= "OTHER"

/note= "the carboxyl group at the C-terminus is replaced by an amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Lys Leu Leu Leu Leu Leu Lys Leu Leu Leu Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 23

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 4, 8, 10)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:12
(D) OTHER INFORMATION:/product= "OTHER"
/note= "the carboxyl group at the C-terminus is replaced by an amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Lys Leu Leu Leu Lys Leu Leu Leu Lys Leu Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 24

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 4, 8, 10)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:12
(D) OTHER INFORMATION:/product= "OTHER"

/note= "the carboxyl group at the C-terminus is replaced by an amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Lys Leu Leu Leu Lys Leu Lys Leu Lys Leu Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 25

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 4, 8, 10)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:12
(D) OTHER INFORMATION:/product= "OTHER"
/note= "the carboxyl group at the C-terminus is replaced by an amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Lys Lys Leu Leu Lys Leu Lys Leu Lys Leu Lys Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 26

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 2, 5, 6, 7, 9, 11, 12)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:12
(D) OTHER INFORMATION:/product= "OTHER"

/note= "the carboxyl group at the C-terminus is replaced by an amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Lys Leu Leu Leu Lys Leu Leu Leu Lys Leu Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 27

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 2, 5, 6, 7, 9, 11, 12)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:12
(D) OTHER INFORMATION:/product= "OTHER"
/note= "the carboxyl group at the C-terminus is replaced by an amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Lys Leu Leu Leu Lys Leu Lys Leu Lys Leu Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 28

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 4)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Lys Leu Leu Leu Leu Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 29

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 4, 6)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Lys Leu Leu Leu Lys Leu Leu Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 30

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 6, 8, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Lys Leu Leu Leu Lys Leu Lys Leu Lys Leu Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 31

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 8, 10, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Lys Leu Leu Leu Lys Leu Lys Leu Lys Leu Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 32

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 5, 8, 9, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Lys Leu Leu Leu Lys Leu Lys Leu Lys Leu Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 33

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 4, 8, 10)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:12
(D) OTHER INFORMATION:/product= "OTHER"
/note= "the carboxyl group at the C-terminus is replaced by an amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Lys Ala Ala Ala Lys Ala Ala Ala Lys Ala Ala Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 34

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 4, 8, 10)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:12
(D) OTHER INFORMATION:/product= "OTHER"
/note= "the carboxyl group at the C-terminus is replaced by an amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Lys Val Val Val Lys Val Val Val Lys Val Val Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 35

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 6, 8, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Lys Val Val Val Lys Val Lys Val Lys Val Val Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 36

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1..4, 8, 10, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

Lys Val Val Val Lys Val Lys Val Lys Val Val Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 37

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 5, 8, 9, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

Lys Val Val Val Lys Val Lys Val Lys Val Val Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 38

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 6)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

Lys Leu Ile Leu Lys Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 39

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 6)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

Lys Val Leu His Leu Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 40

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 6)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

Leu Lys Leu Arg Leu Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 41

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:4
(D) OTHER INFORMATION:/product= "D-amino acid residue"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

Lys Pro Leu His Leu Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 42

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 6)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

Lys Leu Ile Leu Lys Leu Val Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 43

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 4, 5, 6)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

Lys Val Phe His Leu Leu His Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 44

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 2, 4, 6, 7)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

His Lys Phe Arg Ile Leu Lys Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 45

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:5
(D) OTHER INFORMATION:/product= "D-amino acid residue"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

Lys Pro Phe His Ile Leu His Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 46

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 2, 6, 8, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

Lys Ile Ile Ile Lys Ile Lys Ile Lys Ile Ile Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 47

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 8, 10, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

Lys Ile Ile Ile Lys Ile Lys Ile Lys Ile Ile Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 48

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 3, 5, 8, 9, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

Lys Ile Ile Ile Lys Ile Lys Ile Lys Ile Ile Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 49

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:7
(D) OTHER INFORMATION:/product= "D-amino acid recidue"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

Lys Ile Pro Ile Lys Ile Lys Ile Lys Ile Pro Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 50

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(6, 10)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

Lys Ile Pro Ile Lys Ile Lys Ile Lys Ile Val Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 51

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 4, 6, 8, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

Arg Ile Ile Ile Arg Ile Arg Ile Arg Ile Ile Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 52

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 6, 7, 8, 10, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

Arg Ile Ile Ile Arg Ile Arg Ile Arg Ile Ile Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 53

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 3, 5, 8, 9, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

Arg Ile Ile Ile Arg Ile Arg Ile Arg Ile Ile Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 54

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 6, 8, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

Arg Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 55

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 8, 10, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

Arg Ile Ile Val Arg Ile Arg Leu Arg Ile Ile Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 56

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 5, 8, 9, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

Arg Ile Gly Ile Arg Leu Arg Val Arg Ile Ile Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 57

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 6, 8, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57:

Lys Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 58

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 8, 10, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58:

Arg Ile Ala Val Lys Trp Arg Leu Arg Phe Ile Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 59

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 5, 8, 9, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59:

Lys Ile Gly Trp Lys Leu Arg Val Arg Ile Ile Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 60

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 4, 7, 10, 11)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60:

Lys Lys Ile Gly Trp Leu Ile Ile Arg Val Arg Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 61

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 6, 8, 11, 13)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61:

Arg Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg Ile Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 62

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1..4, 8, 10, 11, 13, 14)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62:

Arg Ile Ile Val Arg Ile Arg Leu Arg Ile Ile Arg Val Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 63

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 5, 8, 9, 11, 14)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 63:

Arg Ile Gly Ile Arg Leu Arg Val Arg Ile Ile Arg Arg Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 64

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 2, 6, 7, 11, 15, 16)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 64:

Lys Ile Val Ile Arg Ile Arg Ala Arg Leu Ile Arg Ile Arg Ile Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 65

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 7, 8, 10, 11, 13, 14, 16)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 65:

Arg Ile Ile Val Lys Ile Arg Leu Arg Ile Ile Lys Lys Ile Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 66

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 3, 5, 8, 9, 11, 13, 14, 16)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 66:

Lys Ile Gly Ile Lys Ala Arg Val Arg Ile Ile Arg Val Lys Ile Ile
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 67

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 7, 8, 10, 11, 13, 14, 16)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 67:

Arg Ile Ile Val His Ile Arg Leu Arg Ile Ile His His Ile Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 68

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 3, 5, 8, 9, 11, 13, 14, 16)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 68:

His Ile Gly Ile Lys Ala His Val Arg Ile Ile Arg Val His Ile Ile
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 69

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 4, 7, 9, 10, 11, 13, 14, 16)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 69:

Arg Ile Tyr Val Lys Ile His Leu Arg Tyr Ile Lys Lys Ile Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 70

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 3, 5, 8, 9, 11, 13, 14, 16)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 70:

Lys Ile Gly His Lys Ala Arg Val His Ile Ile Arg Tyr Lys Ile Ile
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 71

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 7, 8, 10, 11, 13, 14, 16)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 71:

Arg Ile Tyr Val Lys Pro His Pro Arg Tyr Ile Lys Lys Ile Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 72

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 5, 8, 9, 11, 13, 14, 16)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 72:

Lys Pro Gly His Lys Ala Arg Pro His Ile Ile Arg Tyr Lys Ile Ile
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 73

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 6, 7, 11, 15, 16, 18)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 73:

Lys Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg Ile Arg Ile Arg
1 5 10 15

Lys Ile Val

(2) INFORMATION FOR SEQ ID NO: 74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 74

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 6, 7, 8, 10, 11, 13, 14, 16, 17, 19)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 74:

Arg Ile Ile Val Lys Ile Arg Leu Arg Ile Ile Lys Lys Ile Arg Leu
1 5 10 15

Ile Lys Lys

(2) INFORMATION FOR SEQ ID NO: 75:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 75

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 4, 5, 8, 9, 11, 13, 14, 16, 17, 18)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 75:

Lys Ile Gly Trp Lys Leu Arg Val Arg Ile Ile Arg Val Lys Ile Gly
1 5 10 15

Arg Leu Arg

(2) INFORMATION FOR SEQ ID NO: 76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 76

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 6, 7, 11, 15, 16, 18, 20, 24, 25)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 76:

Lys Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg Ile Arg Ile Arg
1 5 10 15

Lys Ile Val Lys Val Lys Arg Ile Arg
20 25

(2) INFORMATION FOR SEQ ID NO: 77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 77

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 6, 7, 8, 10, 11, 13, 14, 16, 17, 19..22, 24, 25)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 77:

Arg Phe Ala Val Lys Ile Arg Leu Arg Ile Ile Lys Lys Ile Arg Leu
1 5 10 15

Ile Lys Lys Ile Arg Lys Arg Val Ile Lys
20 25

(2) INFORMATION FOR SEQ ID NO: 78:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 78

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 4, 5, 8, 9, 11, 13, 14, 16, 17, 18, 22, 23, 24, 27, 28, 30)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 78:

Lys Ala Gly Trp Lys Leu Arg Val Arg Ile Ile Arg Val Lys Ile Gly
1 5 10 15

Arg Leu Arg Lys Ile Gly Trp Lys Lys Arg Val Arg Ile Lys
20 25 30

(2) INFORMATION FOR SEQ ID NO: 79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 79

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2..5, 7, 8, 10, 11, 13, 14, 16)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 79:

Arg Ile Tyr Val Lys Pro His Pro Arg Tyr Ile Lys Lys Ile Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 80:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 80

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 2, 3, 5, 7..11, 13, 14, 16)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 80:

Lys Pro Gly His Lys Ala Arg Pro His Ile Ile Arg Tyr Lys Ile Ile
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 81

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 4..8, 10..13, 15, 16, 18)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 81:

Lys Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg Ile Arg Ile Arg
1 5 10 15

Lys Ile Val

(2) INFORMATION FOR SEQ ID NO: 82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 82

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 6..11, 13, 14, 16, 17, 19)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 82:

Arg Ile Ile Val Lys Ile Arg Leu Arg Ile Ile Lys Lys Ile Arg Leu
1 5 10 15

Ile Lys Lys

(2) INFORMATION FOR SEQ ID NO: 83:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 83

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2..5, 7, 8, 10, 11, 13, 14, 16)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 83:

Arg Ile Tyr Val Ser Lys Ile Ser Ile Tyr Ile Lys Lys Ile Arg Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 84:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 84

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 4..8, 10..13, 15, 16, 18)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 84:

Lys Ile Val Ile Phe Thr Arg Ile Arg Leu Thr Ser Ile Arg Ile Arg
1 5 10 15

Ser Ile Val

(2) INFORMATION FOR SEQ ID NO: 85:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 85

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(1, 2, 3, 5, 7..11, 13, 14, 16)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 85:

Lys Pro Ile His Lys Ala Arg Pro Thr Ile Ile Arg Tyr Lys Met Ile
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 86

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(9, 20, 21)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 86:

Cys Lys Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu
1 5 10 15

Phe Lys Thr Leu Leu Ser Ala Val Cys
20 25

(2) INFORMATION FOR SEQ ID NO: 87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 87

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(10, 21, 22)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 87:

Cys Lys Lys Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro
1 5 10 15

Leu Phe Lys Thr Leu Leu Ser Ala Val Cys
20 25

(2) INFORMATION FOR SEQ ID NO: 88:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 88

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(11, 22, 23)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 88:

Cys Lys Lys Lys Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser
1 5 10 15

Pro Leu Phe Lys Thr Leu Leu Ser Ala Val Cys
20 25

(2) INFORMATION FOR SEQ ID NO: 89:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 89

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 89:

Cys Arg Ile Val Ile Arg Ile Arg Ile Arg Leu Ile Arg Ile Arg Cys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 90:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 90

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(2, 3, 4, 6, 8..12, 14, 15, 17)
(D) OTHER INFORMATION:/product= "D-amino acid recidues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 90:

Cys Lys Pro Gly His Lys Ala Arg Pro His Ile Ile Arg Tyr Lys Ile
1 5 10 15

Ile Cys

(2) INFORMATION FOR SEQ ID NO: 91:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 91

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 4, 5, 7, 8, 9, 11, 12, 14, 15, 17, 18, 20..23, 25, 26)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 91:

Cys Arg Phe Ala Val Lys Ile Arg Leu Arg Ile Ile Lys Lys Ile Arg
1 5 10 15

Leu Ile Lys Lys Ile Arg Lys Arg Val Ile Lys Cys
20 25

(2) INFORMATION FOR SEQ ID NO: 92:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 23C (part of peptide 92)

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 4, 8, 10)
(D) OTHER INFORMATION:/product= "D-amuno acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:13
(D) OTHER INFORMATION:/product= "OTHER"
/note= "the carboxyl group at the C-terminus is replaced by an amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 92:

Lys Leu Leu Leu Lys Leu Leu Leu Lys Leu Leu Lys Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 93:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: peptide 24C (part of peptide 93)

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:group(3, 4, 8, 10)
(D) OTHER INFORMATION:/product= "D-amino acid residues"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:13
(D) OTHER INFORMATION:/product= "OTHER"
/note= "the carboxyl group at the C-terminus is replaced by an amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 93:

Lys Leu Leu Leu Lys Leu Lys Leu Lys Leu Leu Lys Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 94:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: PARDAXIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 94:

Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Gly Gln
20 25 30

Glu

(2) INFORMATION FOR SEQ ID NO: 95:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: Melittin

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:26
(D) OTHER INFORMATION:/product= "OTHER"
/note= "the carboxyl group at the C-terminus is replaced by an amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 95:

Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro Ala Leu
1 5 10 15

Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln
20 25

CLAIMS

1. A non-hemolytic cytolytic agent selected from a peptide, a complex of bundled peptides, a mixture of peptides or a random peptide copolymer, said agent having a selective cytolytic activity manifested in that it has a cytolytic activity on pathogenic cells, being cells which are non-naturally occurring within the body consisting of microbial pathogenic organisms and malignant cells; and it is non-hemolytic, namely it has no cytolytic effect on red blood cells or has a cytolytic effect on red blood cells at concentrations which are substantially higher than that in which it manifests said cytolytic activity, said non-hemolytic cytolytic agent being selected from the group consisting of:

(1) a cyclic derivative of a peptide having a net positive charge which is greater than +1, and comprising both L-amino acid residues and D-amino acid residues, or comprising one or both of L-amino acid residues and D-amino acid residues, and comprising an α-helix breaker moiety;

(2) a peptide comprising both L-amino acid residues and D-amino acid residues, having a net positive charge which is greater than +1, and having a sequence of amino acids such that a corresponding amino acid sequence comprising only L-amino acid residues is not found in nature, and cyclic derivatives thereof;

(3) a complex consisting of a plurality of 2 or more non-hemolytic cytolytic peptides, each peptide having a net positive charge which is greater than +1, and comprising both L-amino acid residues and D-amino acid residues, or comprising one or both of L-amino acid residues and D-amino acid residues and comprising an α-helix breaker moiety, or cyclic derivatives of the foregoing, said peptides being bundled together by the use of a linker molecule covalently bound to each of the peptides;

(4) a mixture consisting of a plurality of 2 or more non-hemolytic cytolytic peptides, each peptide having a net positive charge which is greater than +1, and comprising both L-amino acid residues and D-amino acid residues, or comprising one or both of L-amino acid residues and D-amino acid residues and comprising an α-helix breaker moiety, or cyclic derivatives of the foregoing; and

(5) a random copolymer consisting of different ratios of a hydrophobic, a positively charged and a D-amino acid.

2. The cyclic peptide according to claim 1(1), comprising both D- and L-amino acid residues having a sequence such that a homogeneous open-chain peptide comprising only L- or only D-amino acid residues and having the same amino acid sequence as said peptide, has an α-helix configuration and has a broad spectrum cytolytic activity manifested on a variety of cells.

3. The cyclic peptide according to claim 2, which is a cyclic diastereomer derived from pardaxin or mellitin or from fragments thereof.

4. The cyclic peptide according to claim 3, in which the net positive charge greater than +1 is due to the native amino acid composition, or is attained by neutralization of free carboxyl groups or by the addition of positively charged amino acid residues and/or positively charged chemical groups.

5. The cyclic peptide according to claim 4, which is selected from a cyclic diastereomer of pardaxin or of a fragment thereof to which Lys residues have been added to the N-terminus and/or aminoethylamino groups have been added to the C-terminus.

6. The cyclic peptide according to claim 5, selected from the cyclic pardaxin-derived peptides herein designated peptides **86-88,** of the sequence:

86. Cyclic $K^1[D]P^7\ L^{18}L^{19}$ [1-22]-par of the sequence:

Cys-Lys-Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-Ser-
Ser-Pro-Leu-Phe-Lys-Thr-**Leu-Leu**-Ser-Ala-Val-Cys

87. Cyclic $K^1\ K^2[D]P^7\ L^{18}L^{19}$ [1-22]-par of the sequence:

Cys-Lys-Lys-Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-Ser-
Ser-Pro-Leu-Phe-Lys-Thr-**Leu-Leu**-Ser-Ala-Val-Cys

88. Cyclic $K^1\ K^2K^3\ [D]P^7\ L^{18}L^{19}$ [1-22]-par of the sequence:

Cys-Lys-Lys-Lys-Gly-Phe-Phe-Ala-Leu-Ile-**Pro**-Lys-Ile-Ile-
Ser-Ser-Pro-Leu-Phe-Lys-Thr-**Leu-Leu**-Ser-Ala-Val-Cys

7. The peptide according to claim 1(2), comprising both L-amino acid residues and D-amino acid residues and having a sequence of amino acids such that a corresponding amino acid sequence comprising only L-amino acid residues is not found in nature.

8. The peptide according to claim 7, having the following characteristics:

(a) it is a non-natural synthetic peptide composed of varying ratios of at least one hydrophobic amino acid and at least one positively charged amino acid, and in which sequence at least one of the amino acid residues is a D-amino acid;

(b) the peptide has a net positive charge which is greater than +1; and

(c) the ratio of hydrophobic to positively charged amino acids is such that the peptide is cytolytic to pathogenic cells but does not cause cytolysis of red blood cells.

9. The peptide according to claim 8, wherein the positively charged amino acid is selected from lysine, arginine and histidine, and the hydrophobic amino acid is selected from leucine, isoleucine, glycine, alanine, valine, phenylalanine, proline, tyrosine and tryptophan.

10. The peptide according to claim 9, wherein the net positive charge greater than +1 is due to the amino acid composition or to the addition of positively charged chemical groups, or which hydrophobicity may be decreased by the addition of polar amino acids such as serine, threonine, methionine, asparagine, glutamine and cysteine.

11. The peptide according to claim 10 having at least 6 amino acid residues, in which the hydrophobic amino acid is leucine, alanine or valine, and the positively charged amino acid is lysine.

12. The peptide according to claim 11, being a diastereomer of a 6-mer, 8-mer or 12-mer peptide composed of leucine and lysine, in which at least one third of the sequence is composed of D-amino acids, but excepting the peptide herein designated **23**:

23. Lys-Leu-**Leu-Leu**-Lys-Leu-Leu-**Leu** -Lys-**Leu**-Leu-Lys-NH_2

13. A Leu/Lys diastereomer according to claim 12, selected from the peptides herein designated **24** to **29**, (SEQ ID NO: 24-29, respectively) of the sequence:

24. Lys-Leu-**Leu-Leu**-Lys-Leu-Lys-**Leu** -Lys-**Leu**-Leu-Lys-NH_2

25. Lys-Lys-**Leu-Leu**-Lys-Leu-Lys-**Leu** -Lys-**Leu**-Lys-Lys-NH_2

26. **Lys-Leu**-Leu-Leu-**Lys-Leu-Leu**-Leu -**Lys**-Leu-**Leu-Lys**-NH_2

27. **Lys-Leu**-Leu-Leu-**Lys-Leu-Lys**-Leu-**Lys**-Leu-**Leu-Lys**-NH_2

28. Lys-**Leu**-Leu-**Leu** -Leu-Lys

29. Lys-**Leu**-Leu-**Leu** - Lys-**Leu**-Leu-Lys

14. The cyclic derivative of a non-natural synthetic peptide according to any one of claims 7-13, selected from the peptides herein designated **92-95**, of the sequence:

92. Cyclic Cys Lys Leu **Leu Leu** Lys Leu Leu **Leu** Lys **Leu** Leu Lys Cys

93. Cyclic Cys Lys Leu **Leu Leu** Lys Leu Lys **Leu** Lys **Leu** Leu Lys Cys

94. HN - Lys Leu **Leu Leu** Lys Leu Leu **Leu** Lys **Leu** Leu Lys – CO

95. HN - Lys Leu **Leu Leu** Lys Leu Lys **Leu** Lys **Leu** Leu Lys – CO

15. A complex of bundled peptides according to claim 1(3) consisting of a plurality of 2 or more non-hemolytic cytolytic peptides according to any one of claims 1-14, said peptides being bundled together through a linker molecule covalently bound to each of the peptides.

16. The complex according to claim 15, wherein the bundle is composed of 2 or more, preferably 5, molecules of the same peptide or of different peptides, and the linker is a peptide according to any one of the preceding claims or a commonly used linker.

17. The complex according to claim 16 selected from the bundled Lys/Leu diastereomers herein designated **96** and **97**:

96. $([D]\text{-}L^{3,4,8,10}\text{-}K_4L_8C)_5$ $[D]\text{-}L^{3,4,8,10}\text{-}K_4L_8$ of the sequence:

(Lys-Leu-**Leu-Leu**-Lys-Leu-Leu-**Leu**-Lys-**Leu**-Leu-Lys-Cys-NH_2)$_5$ Lys-Leu-**Leu-Leu**-Lys-Leu-Leu-**Leu** -Lys-**Leu**-Leu-Lys-NH_2

97. ([D]-$L^{3,4,8,10}$-$K_5L_7C)_5$ [D]-$L^{3,4,8,10}$-K_4L_9 of the sequence:

(Lys-Leu-**Leu-Leu**-Lys-Leu-Lys-**Leu**-Lys-**Leu**-Leu-Lys-Cys-$NH_2)_5$ Lys-Leu-**Leu-Leu**-Lys-Leu-Leu-**Leu** -Lys-**Leu**-Leu-Lys-NH_2

18. The mixture according to claim 1(4) consisting of a plurality of 2 or more non-hemolytic cytolytic peptides, wherein the peptides are as defined in any one of claims 1 to 14.

19. The mixture according to claim 18 comprising a mixture of Lys/Leu 12-mer peptide diastereomers.

20. The non-hemolytic cytolytic random copolymer according to claim 1(5), consisting of different ratios of a hydrophobic, a positively charged and a D-amino acid,

21. The non-hemolytic cytolytic random copolymer according to claim 20, composed of lysine, leucine and D-leucine in the ratio 1 : 1 : 1, 2 : 1 : 1 or 3 : 1 : 1 (Mol).

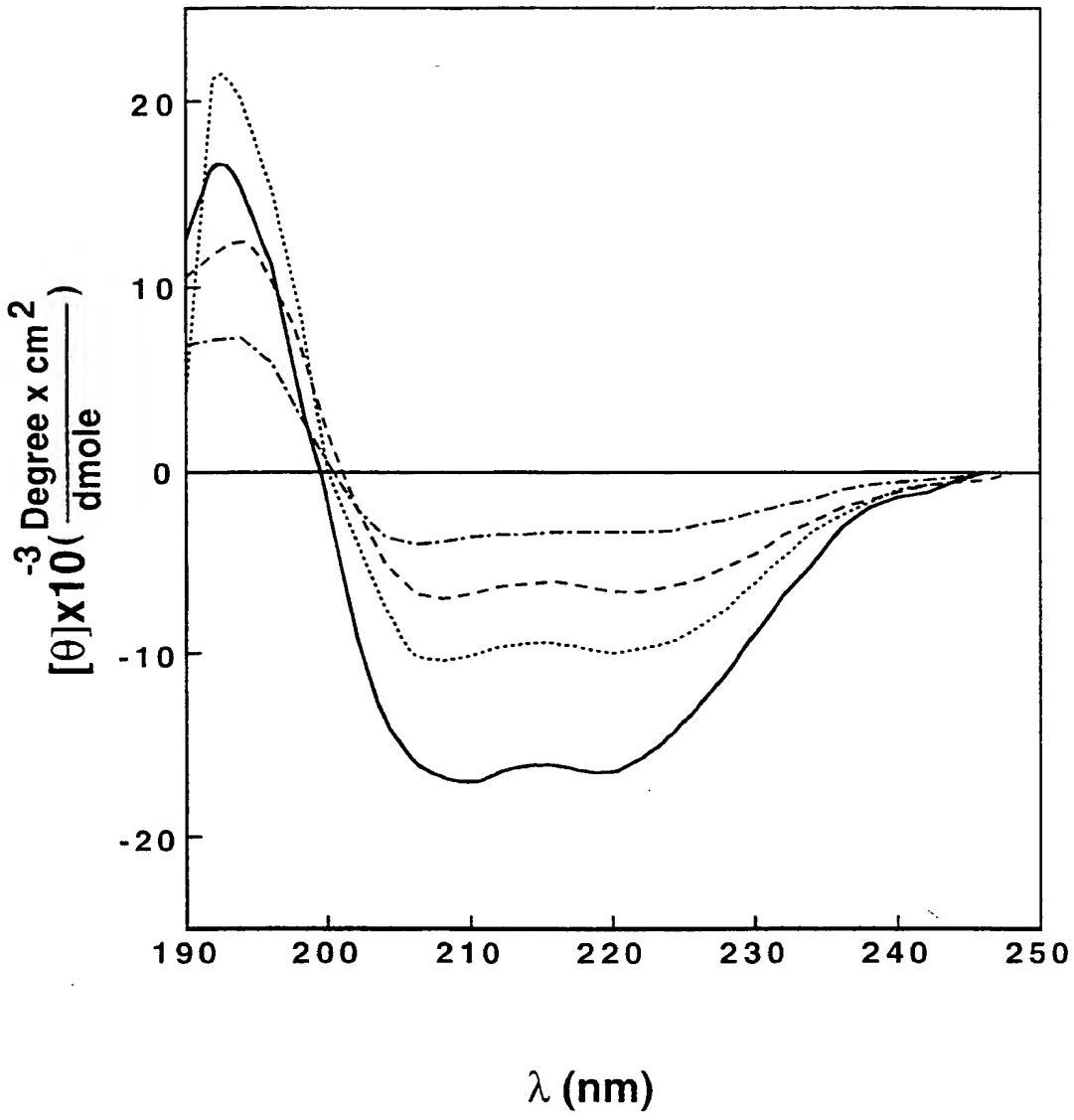
22. A pharmaceutical composition comprising a non-hemolytic cytolytic agent according to any one of claims 1-21 , and a pharmaceutically acceptable carrier.

23. The pharmaceutical composition according to claim 22, for the treatment of infections caused by pathogenic organisms.

24. The pharmaceutical composition according to claim 23, wherein the pathogenic organism is selected from bacteria, fungi, protozoa, mycoplasma and virus.

25. The pharmaceutical composition according to claim 24, wherein the pathogenic organism is a bacterium.

26. The pharmaceutical composition according to claim 22, for the treatment of cancer.


20
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-10
-20
190
200
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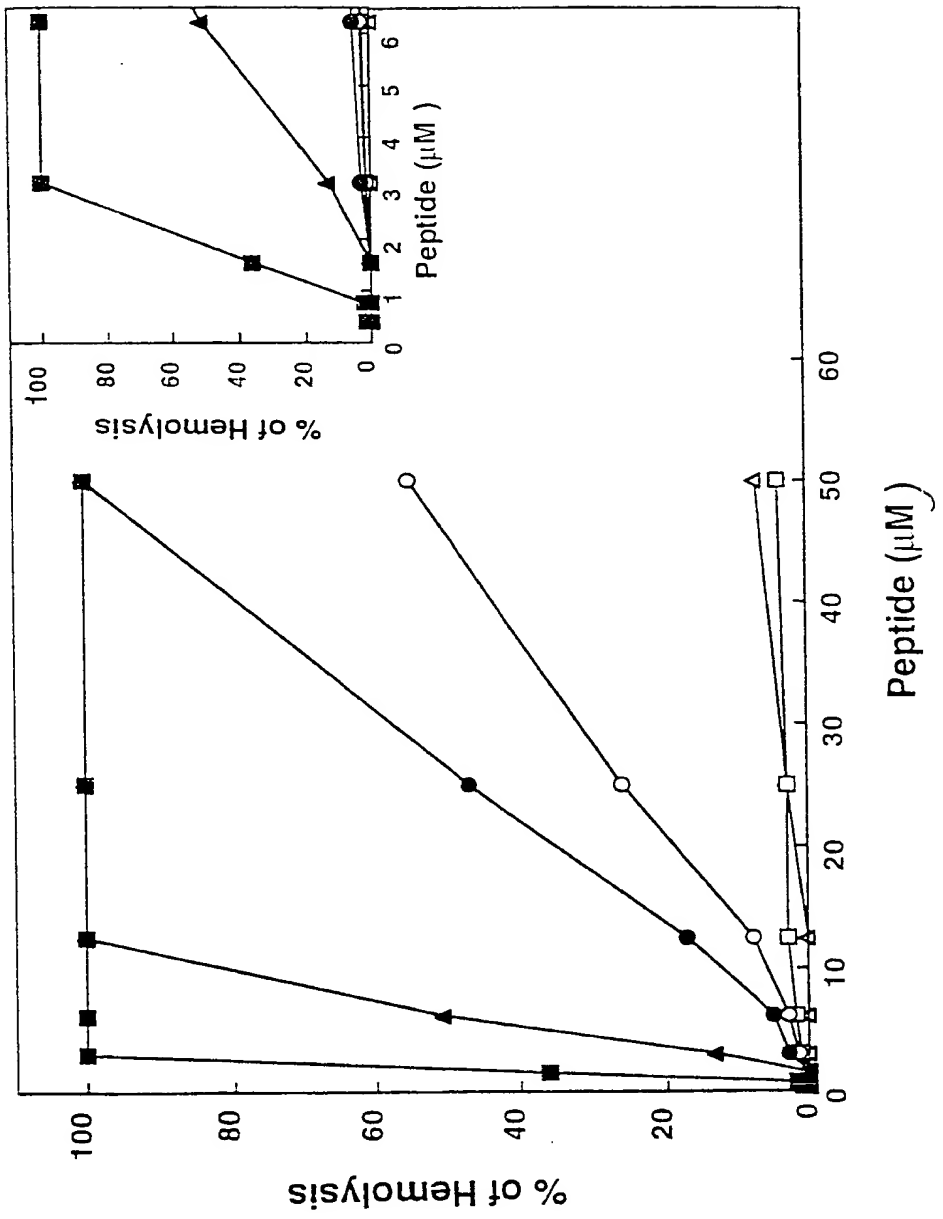
λ (nm)

Fig. 1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60
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1
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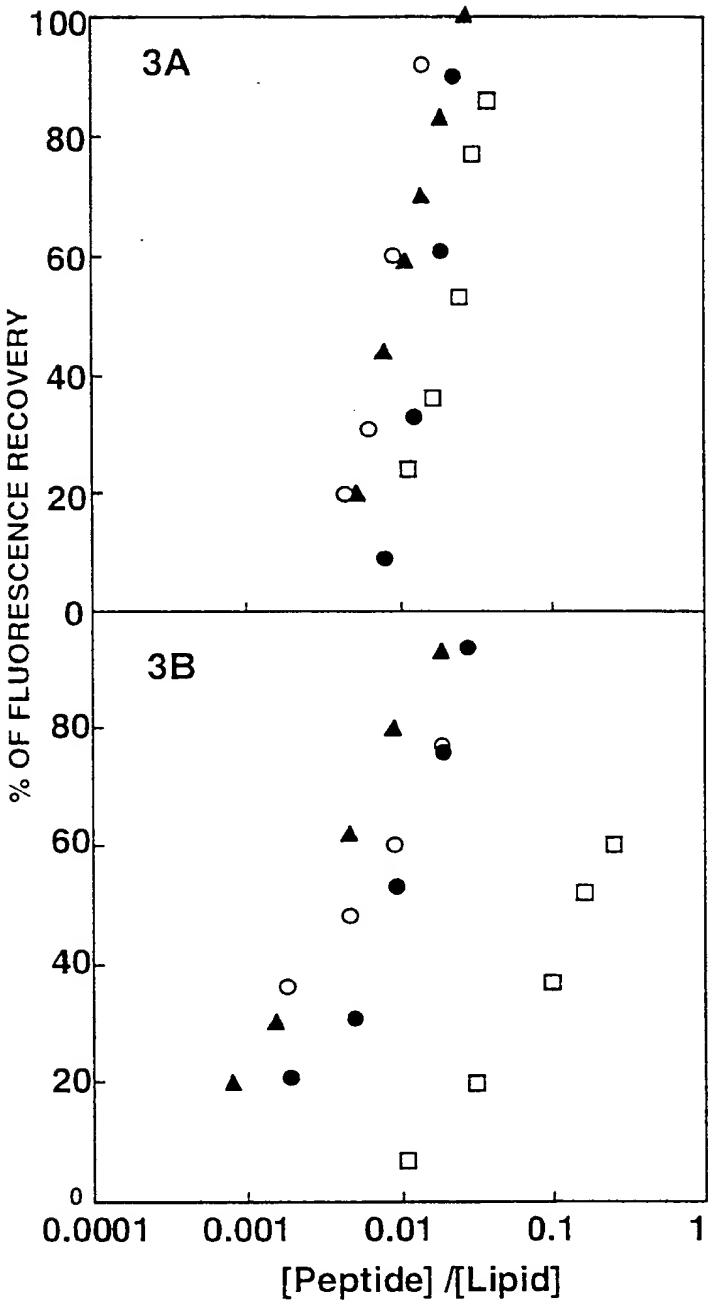
3
4
5
6

Fig. 2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0.1
1
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Fig. 3

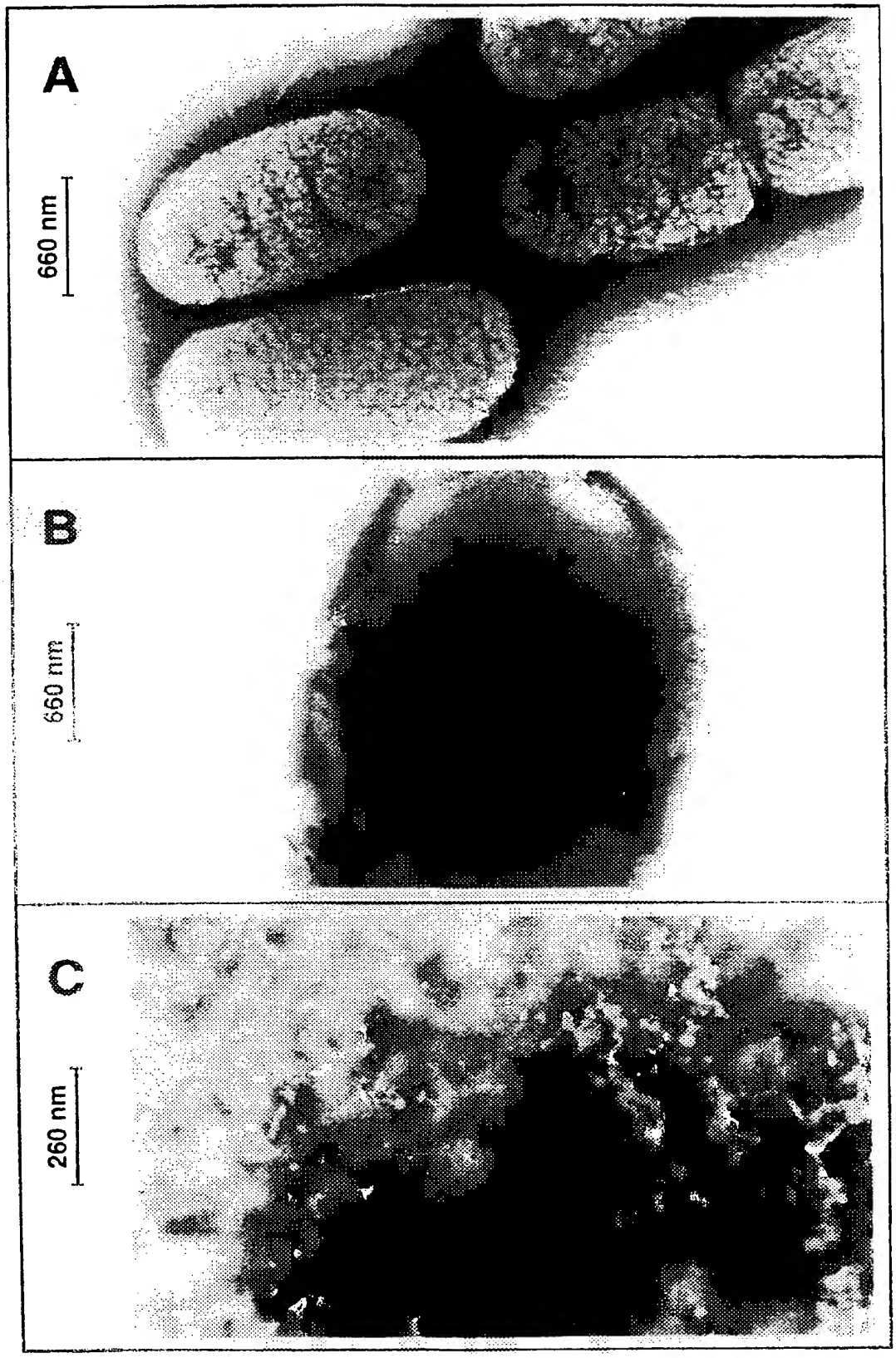
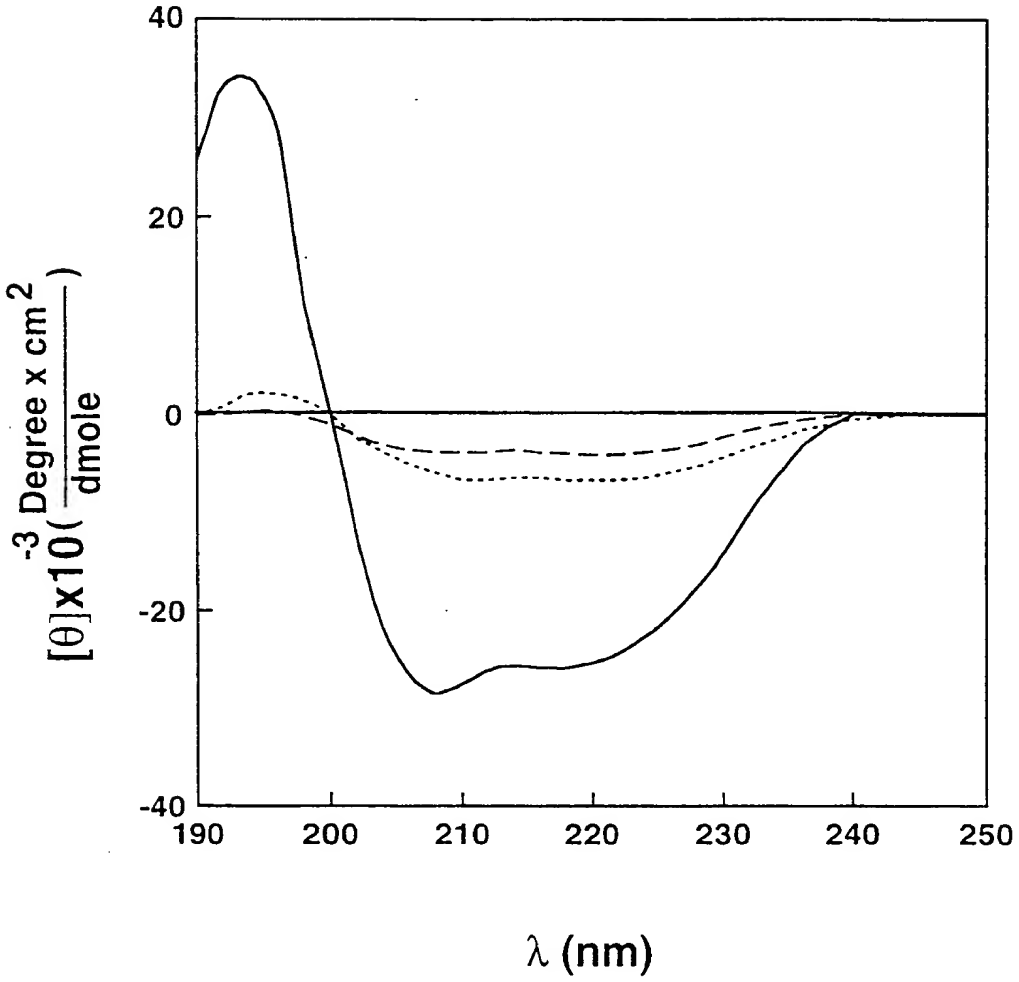


Fig. 4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200
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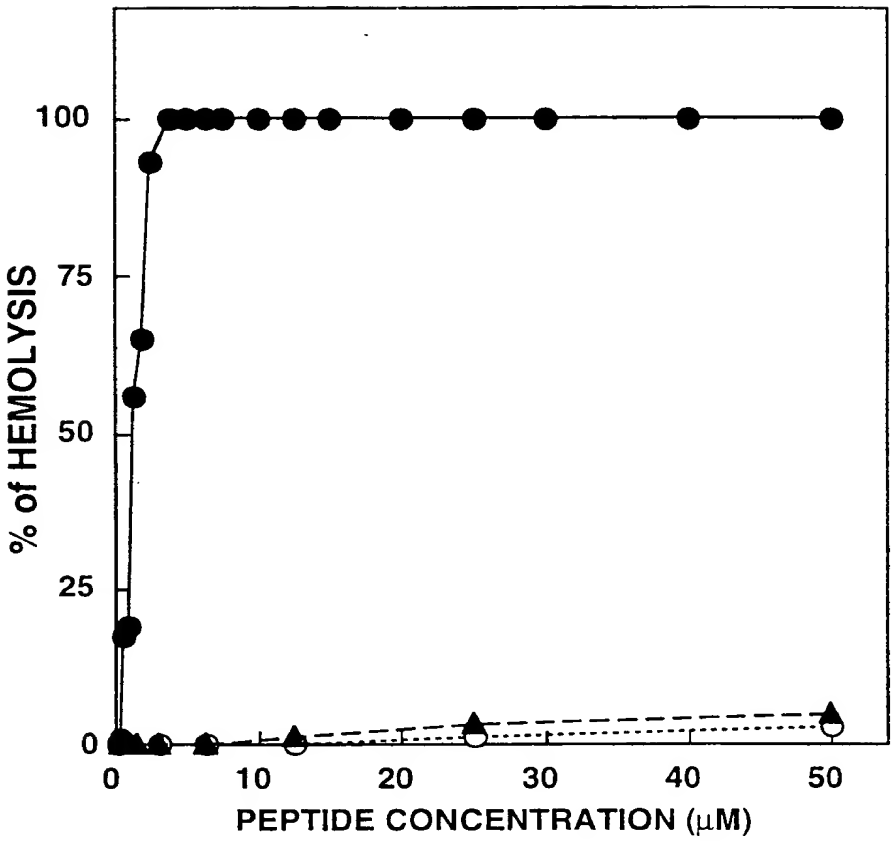
240
250
$[\theta]x10^{-3}$ Degree x cm^2 (dmole)
λ (nm)

Fig. 5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75
50
25
0
% of HEMOLYSIS
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10
20
30
40
50
PEPTIDE CONCENTRATION (μM)

Fig. 6

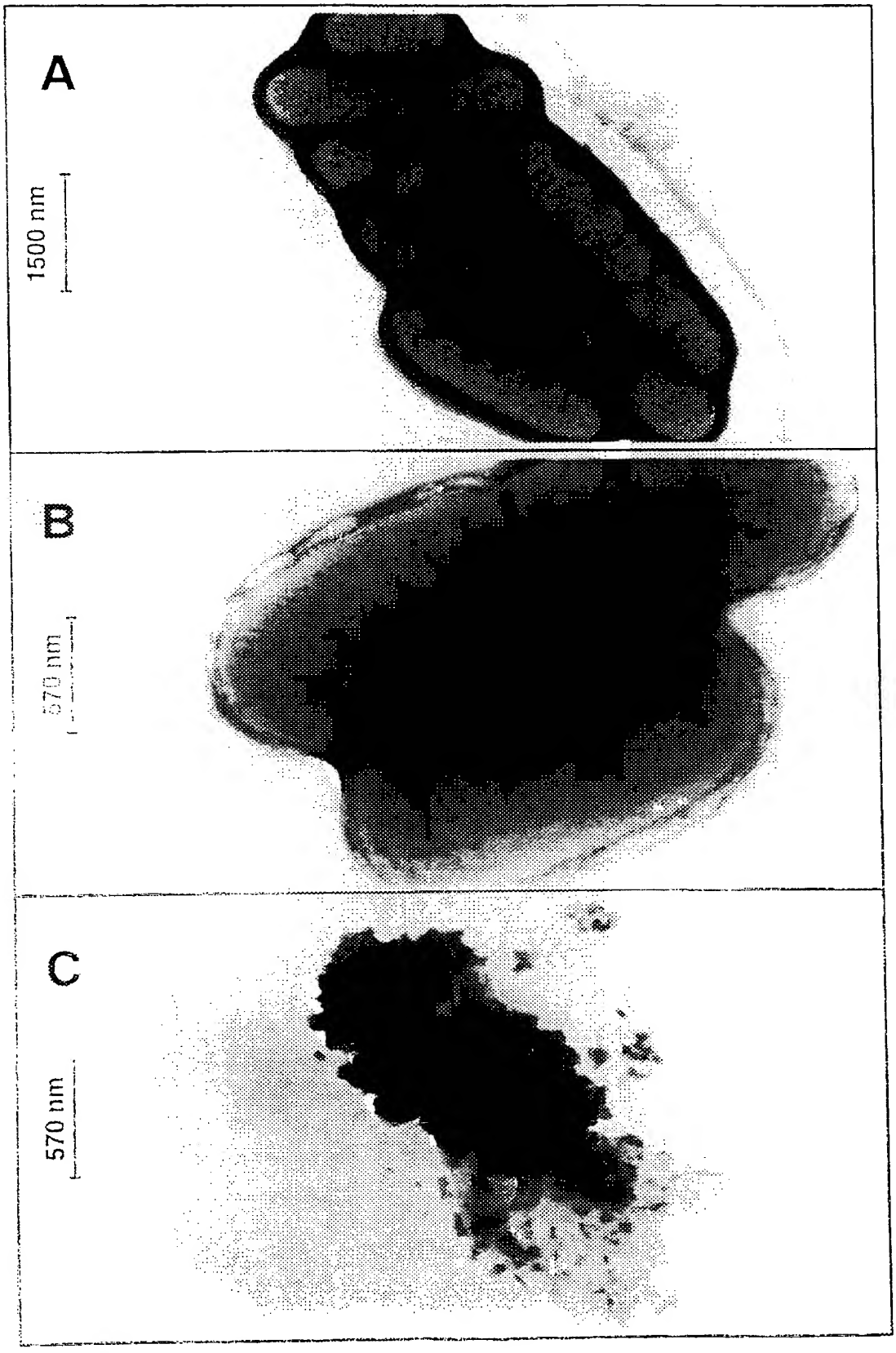
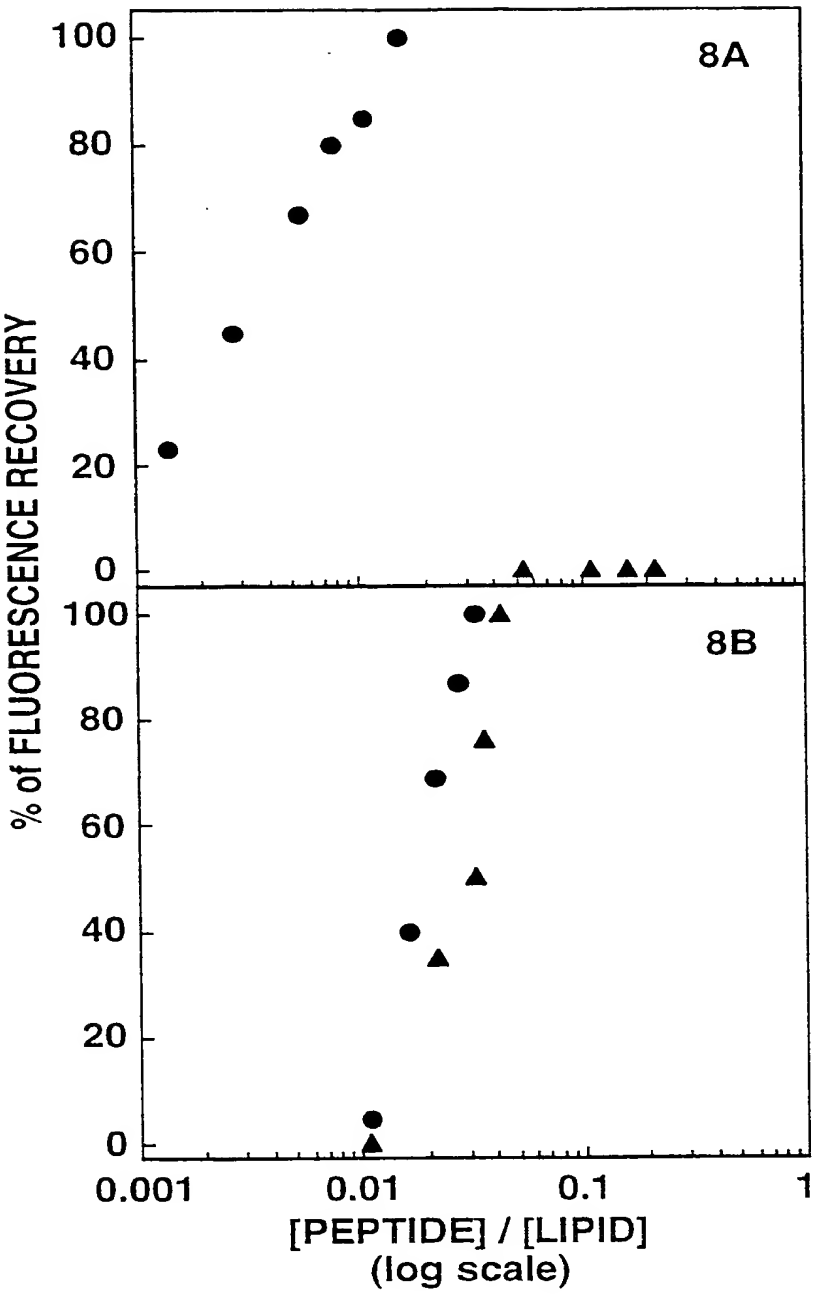


Fig. 7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% of FLUORESCENCE RECOVERY
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20
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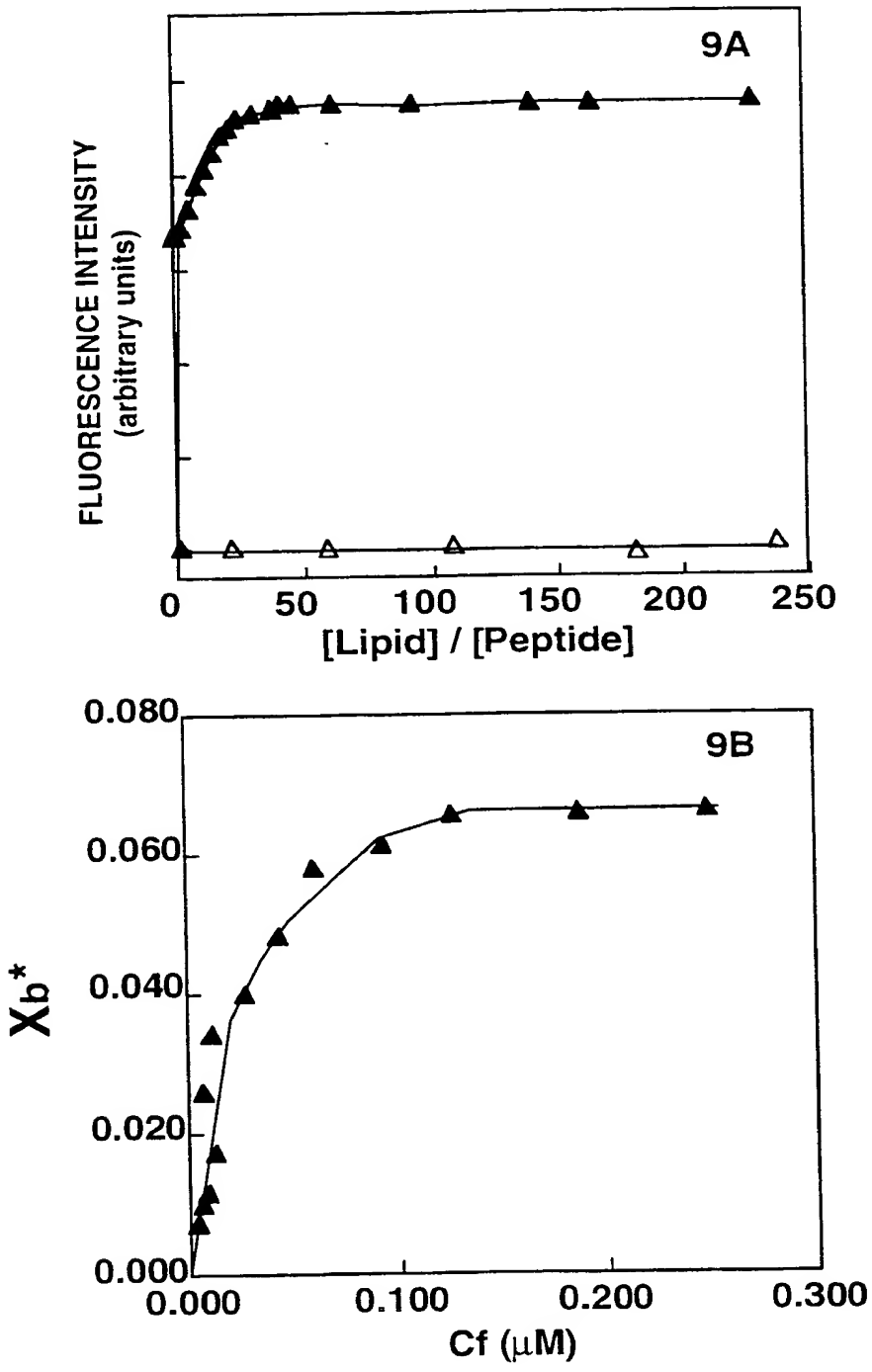
0.01
0.1
1
[PEPTIDE] / [LIPID]
(log scale)

Fig. 8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FLUORESCENCE INTENSITY
(arbitrary units)
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100
150
200
250
[Lipid] / [Peptide]
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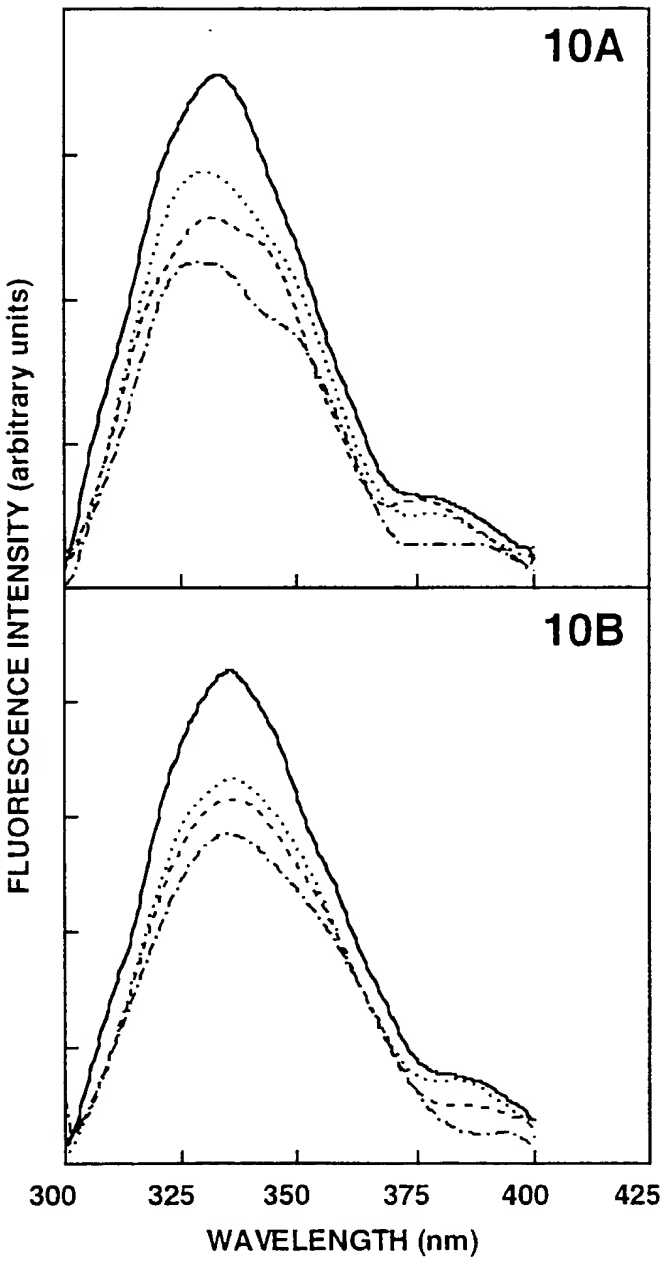
0.020
0.000
Xb*
0.000
0.100
0.200
0.300
Cf (μM)

Fig. 9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FLUORESCENCE INTENSITY (arbitrary units)
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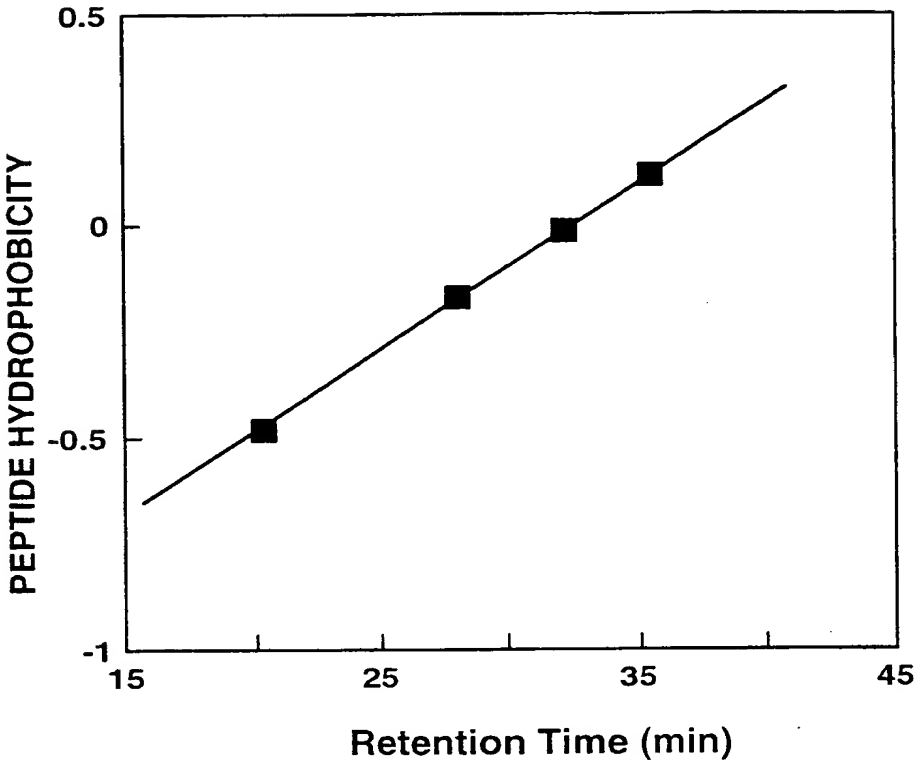
350
375
400
425
WAVELENGTH (nm)

Fig. 10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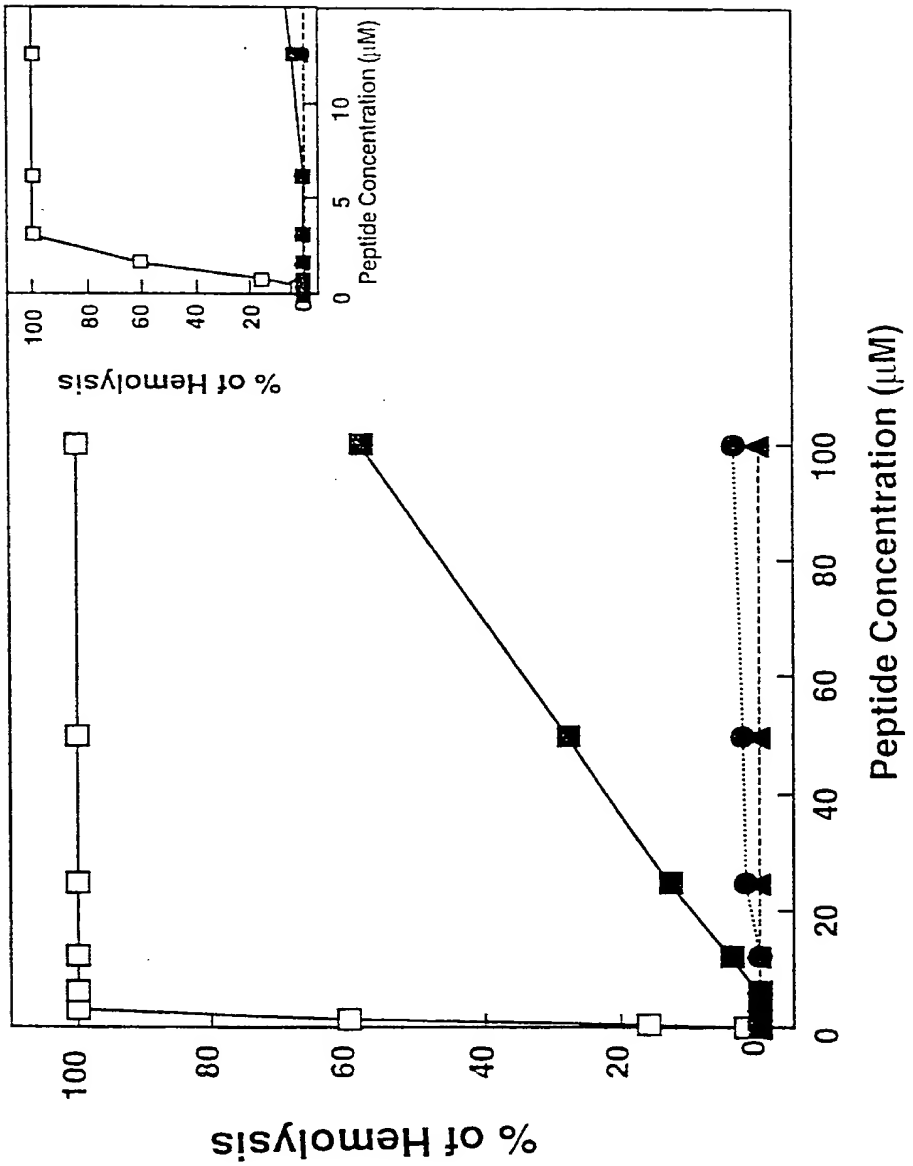
-0.5
-1
PEPTIDE HYDROPHOBICITY
15
25
35
45
Retention Time (min)

Fig. 11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40
60
80
100
Peptide Concentration (μM)
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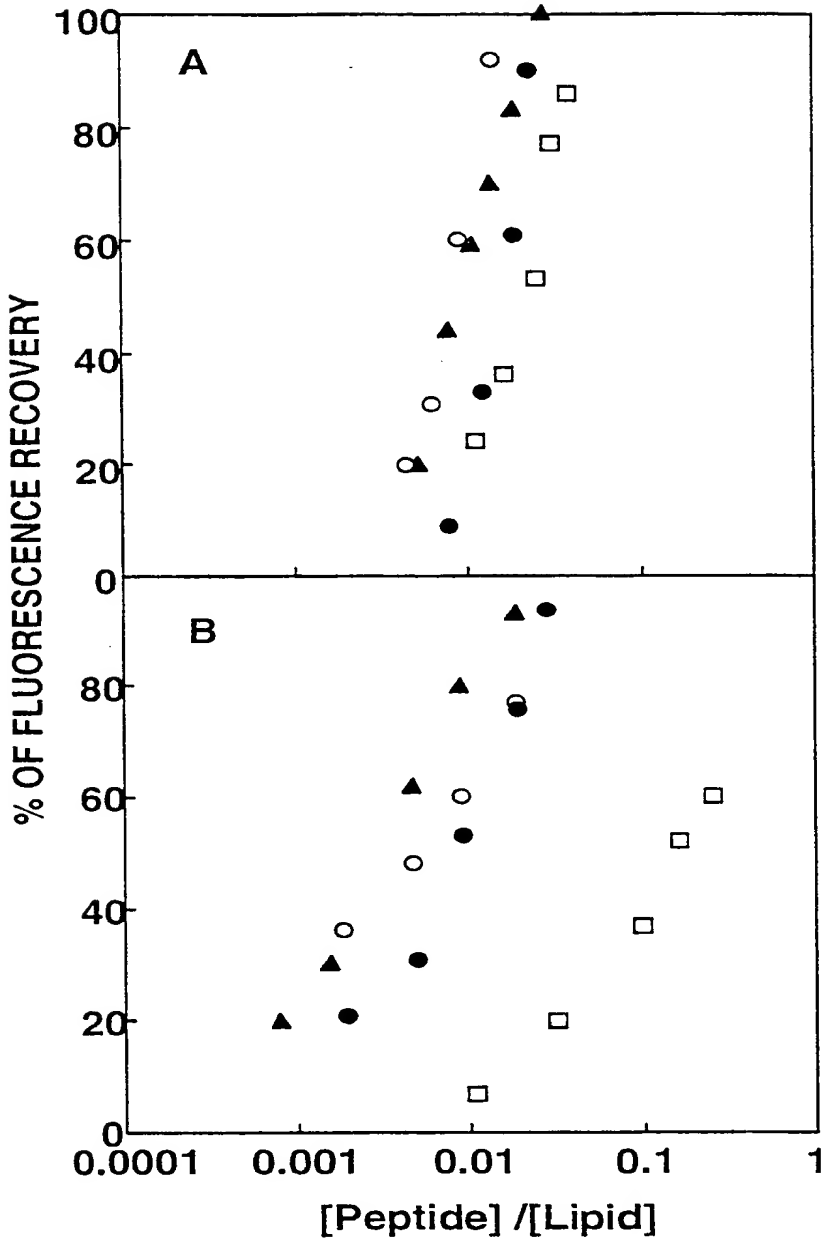
20
40
60
80
100
% of Hemolysis
0
5
10
Peptide Concentration (μM)

Fig. 12


A
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0.01
0.1
1
[Peptide] /[Lipid]

Fig. 13

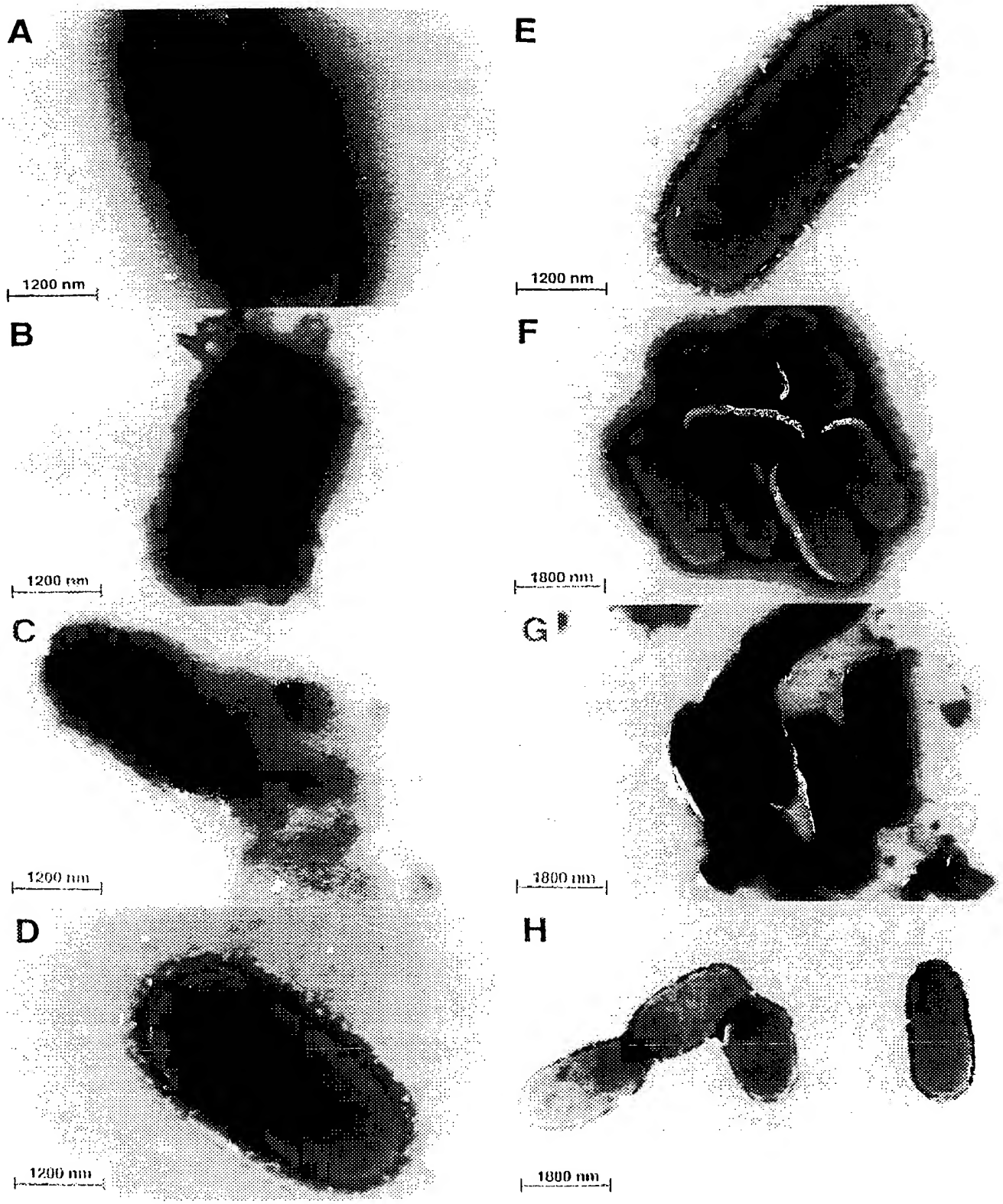
A
1200 nm
B
1200 nm
C
1200 nm
D
1200 nm
E
1200 nm
F
1800 nm
G
1800 nm
H
1800 nm

Fig. 14

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/IL 98/00081

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/00 C07K14/435 C07K14/46 A61K38/04 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Y POUNY & Y SHAI: "Interaction of D-amino acids incorporated analogues of pardaxin with membranes " BIOCHEMISTRY., vol. 31, no. 39, 6 October 1992, EASTON, PA US, pages 9482-9490, XP002036499 see throughout, especially Table 1, example 7 see table 1 --- -/--	1-4, 22-26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

' Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 July 1998	30/07/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Masturzo, P

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 98/00081

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	D RAPAPORT ET AL.: "pH and ionic strength-dependent fusion of phospholipid vesicles induced by pardaxin analogues or by mixtures of charge-reversed peptides" BIOCHEMISTRY., vol. 32, no. 13, 6 April 1993, EASTON, PA US, pages 3291-3297, XP002036498 see table 1 ---	1-4, 22-26
X	Z OREN & Y SHAI: "Selective lysis of bacteria but not mammalian cells by diastereoisomers of melittin: structure-function study" BIOCHEMISTRY., vol. 36, no. 7, 18 February 1997, EASTON, PA US, pages 1826-1835, XP002071935 see the whole document ---	1-4, 22-26
X	Y SHAI & Z OREN: "Diastereoisomers of cytolysins, a novel class of potent antibacterial peptides" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 271, no. 13, 29 March 1996, MD US, pages 7305-7308, XP002036500 see the whole document ---	1-4, 22-26
A	CHEMICAL ABSTRACTS, vol. 124, no. 23, 3 June 1996 Columbus, Ohio, US; abstract no. 314982, Z OREN & Y SHAI: "A class of highly potent antibacterial peptides derived from pardaxin apore-forming peptide isolated from Moses sole fish" XP002071937 & EUR. J. BIOCHEM., vol. 237, no. 1, 1996, pages 303-310, see abstract ---	1-26
P,X	Z OREN ET AL.: "A repertoire of novel antibacterial diastereisomeric peptides with selective cytolytic activity" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 272, no. 23, 6 June 1997, MD US, pages 14643-14649, XP002071936 see the whole document ---	1-26
P,X	WO 97 31019 A (YEDA RESEARCH AND DEVELOPMENT) 28 August 1997 see the whole document -----	1-26

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IL 98/00081

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9731019 A	28-08-1997	AU 1731997 A	10-09-1997

PCT WIPO INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/00, 14/435, 14/46, A61K 38/04, 38/16 — A1

(11) International Publication Number: **WO 98/37090**

(43) International Publication Date: 27 August 1998 (27.08.98)

(21) International Application Number: PCT/IL98/00081

(22) International Filing Date: 19 February 1998 (19.02.98)

(30) Priority Data:
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(34) Countries for which the regional or international application was filed: IL et al.

(71) Applicant *(for all designated States except US)*: YEDA RESEARCH AND DEVELOPMENT CO. LTD. [IL/IL]; Weizmann Institute of Science, P.O. Box 95, 76100 Rehovot (IL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SHAI, Yechiel [IL/IL]; Bar Simantov 70, 56333 Yahud (IL). OREN, Ziv [IL/IL]; Harav Kook Street 3, 75306 Rishon Le-Zion (IL).

(74) Agent: BEN-AMI, Paulina; Yeda Research and Development Co. Ltd., Weizmann Institute of Science, P.O. Box 95, 76100 Rehovot (IL).

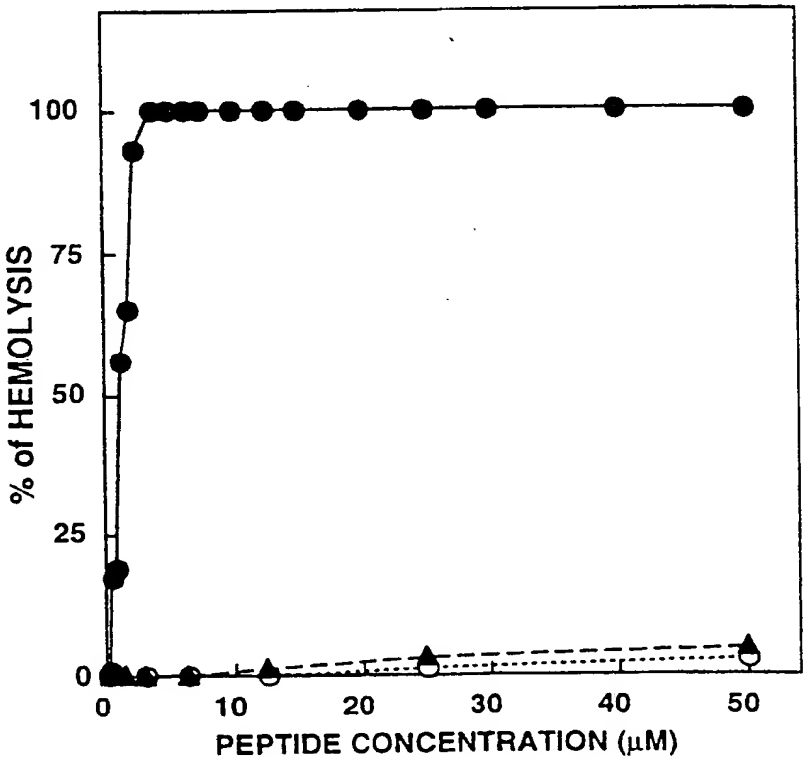
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANTIPATHOGENIC SYNTHETIC PEPTIDES AND COMPOSITIONS COMPRISING THEM

(57) Abstract

Non-hemolytic cytolytic agents selected from peptides, complexes of bundled peptides, mixtures of peptides or random peptide copolymers have a selective cytolytic activity manifested in that they have a cytolytic activity on pathogenic cells, being cells which are non-naturally occurring within the body consisting of microbial pathogenic organisms and malignant cells; and are non-hemolytic, having no cytolytic effect on red blood cells. The peptides may be cyclic derivatives of natural peptides such as pardaxin and mellitin and fragments thereof in which L-amino acid residues are replaced by corresponding D-amino acid residues, or are diastereomers of linear peptides composed of varying ratios of at least one positively charged amino acid and at least one hydrophobic amino acid, and in which at least one of the amino acid residues is a D-amino acid. Pharmaceutical compositions comprising the non-hemolytic cytolytic agents can be used for the treatment of several diseases caused by pathogens including antibacterial, fungal, viral, mycoplasma and protozoan infections and for the treatment of cancer.



INTERNATIO SEARCH REPORT

Int [redacted] Application No
PCT/IL 98/00081

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/00 C07K14/435 C07K14/46 A61K38/04 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Y POUNY & Y SHAI: "Interaction of D-amino acids incorporated analogues of pardaxin with membranes " BIOCHEMISTRY., vol. 31, no. 39, 6 October 1992, EASTON, PA US, pages 9482-9490, XP002036499 see throughout, especially Table 1, example 7 see table 1 --- -/--	1-4, 22-26

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 July 1998	30/07/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	D RAPAPORT ET AL.: "pH and ionic strength-dependent fusion of phospholipid vesicles induced by pardaxin analogues or by mixtures of charge-reversed peptides" BIOCHEMISTRY., vol. 32, no. 13, 6 April 1993, EASTON, PA US, pages 3291-3297, XP002036498 see table 1 ---	1-4, 22-26
X	Z OREN & Y SHAI: "Selective lysis of bacteria but not mammalian cells by diastereoisomers of melittin: structure-function study" BIOCHEMISTRY., vol. 36, no. 7, 18 February 1997, EASTON, PA US, pages 1826-1835, XP002071935 see the whole document ---	1-4, 22-26
X	Y SHAI & Z OREN: "Diastereoisomers of cytolysins, a novel class of potent antibacterial peptides" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 271, no. 13, 29 March 1996, MD US, pages 7305-7308, XP002036500 see the whole document ---	1-4, 22-26
A	CHEMICAL ABSTRACTS, vol. 124, no. 23, 3 June 1996 Columbus, Ohio, US: abstract no. 314982, Z OREN & Y SHAI: "A class of highly potent antibacterial peptides derived from pardaxin apore-forming peptide isolated from Moses sole fish" XP002071937 & EUR. J. BIOCHEM., vol. 237, no. 1, 1996, pages 303-310, see abstract ---	1-26
P,X	Z OREN ET AL.: "A repertoire of novel antibacterial diastereisomeric peptides with selective cytolytic activity" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 272, no. 23, 6 June 1997, MD US, pages 14643-14649, XP002071936 see the whole document ---	1-26
P,X	WO 97 31019 A (YEDA RESEARCH AND DEVELOPMENT) 28 August 1997 see the whole document -----	1-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ati pplication No
PCT/IL 98/00081

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9731019 A	28-08-1997	AU 1731997 A	10-09-1997

ENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
9610 PCT 2		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IL 98/00081	19/02/1998	20/02/1997
Applicant		
YEDA RESEARCH AND DEVELOPMENT CO. LTD. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [X] filed with the international application.
   - [ ] furnished by the applicant separately from the international application.
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:
   Figure No. 6
   - [ ] as suggested by the applicant.
   - [X] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

Int[redacted]al Application No
PCT/IL 98/00081

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/00 C07K14/435 C07K14/46 A61K38/04 A61K38/16

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
17 July 1998	30/07/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document. with indication.where appropriate, of the relevant passages	Relevant to claim No.
X	D RAPAPORT ET AL.: "pH and ionic strength-dependent fusion of phospholipid vesicles induced by pardaxin analogues or by mixtures of charge-reversed peptides" BIOCHEMISTRY., vol. 32, no. 13, 6 April 1993, EASTON, PA US, pages 3291-3297, XP002036498 see table 1 ---	1-4, 22-26
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X	Y SHAI & Z OREN: "Diastereoisomers of cytolysins, a novel class of potent antibacterial peptides" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 271, no. 13, 29 March 1996, MD US, pages 7305-7308, XP002036500 see the whole document ---	1-4, 22-26
A	CHEMICAL ABSTRACTS, vol. 124, no. 23, 3 June 1996 Columbus, Ohio, US; abstract no. 314982, Z OREN & Y SHAI: "A class of highly potent antibacterial peptides derived from pardaxin apore-forming peptide isolated from Moses sole fish" XP002071937 & EUR. J. BIOCHEM., vol. 237, no. 1, 1996, pages 303-310, see abstract ---	1-26
P,X	Z OREN ET AL.: "A repertoire of novel antibacterial diastereisomeric peptides with selective cytolytic activity" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 272, no. 23, 6 June 1997, MD US, pages 14643-14649, XP002071936 see the whole document ---	1-26
1 P,X	WO 97 31019 A (YEDA RESEARCH AND DEVELOPMENT) 28 August 1997 see the whole document -----	1-26

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IL 98/00081

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9731019 A	28-08-1997	AU 1731997 A	10-09-1997

PATENT COOPERATION TREATY

19

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
9610 PCT 2		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL98/00081	19/02/1998	20/02/1997
International Patent Classification (IPC) or national classification and IPC C07K14/00		
Applicant YEDA RESEARCH AND DEVELOPMENT CO. LTD. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
14/09/1998	2.8.05.99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. (+49-89) 2399-0 Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer Pilat, D Telephone No. (+49-89) 2399 8668

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL98/00081

I. Basis of th r port

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-102 as originally filed

Claims, No.:

1-26 as originally filed

Drawings, sheets:

1/14-14/14 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whos priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5,6,12-17,20-26
	No:	Claims	1-4,7-11,18
Inventive step (IS)	Yes:	Claims	12-14,17
	No:	Claims	5,6,15,16,20-26
Industrial applicability (IA)	Yes:	Claims	1-26
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Ad Section I: Basis of the opinion

1) Reference is made to the following documents:

D1 Y Pouny & Y. Shai Biochemistry vol.31, n°39 6 October 1992 p.9482-90
D2 D. Rapaport et al. Biochemsitry vol.32, n°13 6 April 1993, p.3291-97
D3 Z. Oren & Y. Shai Biochemistry vol. 36, n°7 18 February 1997, p.1826-35
D4 Y. Shai & Z. Oren Journal of Biological Chemistry vol.271, n°13 29 March 1996, p.7305-8

Ad Section II :Priority

2) Priority (Article 8 PCT)

The priority document pertaining to the present application was not available at the time of establishing this IPER. Hence, it is based on the assumption that all claims enjoy priority rights from the filing date of the priority document. If it later turns out that this is not correct, the P documents cited in the international search report could become relevant to assess whether all claims satisfy the criteria set forth in Article 33(1) PCT.

Ad Section V :Reasoned statement under Rule 66.2(a)(ii); citations and explanations supporting such statement

3) Novelty (Article 33 (2) PCT)

3.1 D1 describes a set of Pardaxin analogues among which diastereoisomers (see table I). From this table only pardaxin D-$L^{18}L^{19}$-Par and D-P^{13}-Par were able to lyse the erythrocytes all the other were non-hemolytic (see p.9488, col.1 last full paragraph).
D2 describes pardaxin analogues in which amino groups were substituted by carboxylic groups (see table I).
D3 describes diastereoisomers of melittin (see table I). It states also that pardaxin and melittin have a net charge of +1 and +6 respectively (see p.1827, col.1 lines 12-20), that pardaxin is 40-100 fold less hemolytic than melittin towards human

erythrocytes and that they are both potent antibacterial peptides against Gram-positive and Gram-negative bacteria (see p.1827, col.1 lines 30-33). This document shows also that diastereoisomers peptides in the absence of an alpha helical structure caused abrogation of their cytotoxic effects on mammalian cells, although they retained antibacterial activity (see p.1827, col.1, lines 37-41 and p.1827, col.1 last paragraph and p.1832 col.2 lines 16-25).
Based on all these findings, D3 suggests designing antibacterial peptides, which lack pathological and pharmalogical effects induced by alpha-helical lytic cytolysin and proposed the synthesis of diastereoisomers of pardaxin which do not exert these activities. Furthermore, D-amino acid local substitution should enable a controlled clearance of the antibacterial peptides. Finally, D3 suggests that modulating both the hydrophobicity and net charge of diastereoisomers of linear cytotoxic polypeptides may be sufficient to design potent antibacterial polypeptides for the treatment of infectious diseases (see p.1834, col.1 last paragraph).
D4 discloses a set of pardaxin peptides, among which Tapar. This peptide has a net charge of +5 and a C-terminus converted to a positive one by transamination. Three diastereoisomers of Tapar have been synthesized (see table I and p.7306, col.1 "Results" first paragraph) and reduced the hemolytic properties of these analogues, whereas they all retained antibacterial activity (see p.7306, col.2 lines 24-27 and lines 32-35; p.7307 col.2 lines 5-18).

In view of the teaching of these documents, the subject-matter of claims 1 to 4 which comprises a non-hemolytic cytolytic agent having a selective cytolytic activity on pathogenic or malignant cells, is non-hemolytic or has a reduced hemolytic property, is a peptide comprising both L-amino acid residues and D-amino acid residues, has a net positive charge greater than +1 and has a sequence not found in nature is anticipated by the peptides n°6-13 in D1 table I; by peptide n°9 D2 table I (spontaneous succinylation is not found in nature); by the second peptide in D3 table I (transamination is very unlikely to occur in nature) and by the second to forth peptides in D4 table I (transamination is very unlikely to occur in nature). The same objection applies to a mixture of cytolytic peptides (see also D2, Fig.4). Thus claims 1 to 4 and 7 to 11 and 18 lack novelty.

3.2 None of the cited prior art discloses a cyclic pardaxin or a peptide as claimed in

claims 5, 6, 12-17 and 22-26. Thus, these claims seem to be novel.

4) Inventive step (Article 33 (3) PCT)

4.1 D2 describes the use of aminoethylamino groups (see D2, Table I peptide n°8 and 11). Furthermore, it is known that modification of the net charge may lead to a potent antibacterial polypeptides for the treatment of infectious diseases (D3 see p.1834, col.1 last paragraph). Therefore it appears trivial to add such an amino group to a selected peptide diastereoisomer in order to potentiate or lower its lytic efficiency. Thus, claim 5 lacks an inventive step.

4.2 Linear peptide of pardaxin are known (see point 3.1 above). Similarly the synthesis of cyclic peptides are also known in the art. Therefore to synthesize a cyclic form of pardaxin in order to provide an alternative to the linear peptide does not seem to be inventive unless the applicant shows a surprising technical effect associated with this particular form. At present claim 6 lacks an inventive step.

4.3 It is general knowledge that complex of peptides may be stabilized by a covalent linker molecule. In the light of the results obtained in D2 which shows that there is aggregation between peptides (Abstract, last sentence) the skilled person would have linked these diastereoisomers monomers together in order to stabilize them without any inventive activity. As a consequence claim 15 and 16 lack an inventive step.

4.4 The synthesis of a copolymer of a known peptide (see point 3.1 above) is an obvious alternative to the known monomers. The further selection of a copolymer having different ratios of hydrophobic, a positively charged and a D amino acid or a specific ratio seems to relate to an arbitrary choice the skilled person would have considered, since it was known that modulation of the hydrophobicity and net charge of diastereoisomers of linear cytotoxic polypeptides allows the design of potent antibacterial polypeptides (see D3 p.1834, col.1 last paragraph). Claims 20 and 21 are obvious.

4.5 Since the cytolytic peptides claimed in claim 1 are known and the application of these peptides for therapy, treatment of infectious diseases and against bacteria

are suggested (D3, p.1834, col.1, last paragraph) no inventive step can be seen for pharmaceutical composition containing such compounds. Therefore claims 22 to 26 are obvious.

4.6 The subject-matter of claim 12 which refer to a 6 mer, 8-mer, 12-mer peptide composed of leucine and lysine in which at least one third of the sequence is composed of D-amino acids seems to be new and is not suggested in the cited prior art when considered alone or in any combination. Consequently claim 12 as well as dependent claims 13 and 14 seems to involve an inventive step. For the same reasons claim 17 seems also to enjoy an inventive step.

Ad Section VIII : Certain observations on the international application.

5) Clarity (Article 6 PCT)

As defined in Article 6 PCT the claims shall **define** the matter for which protection is sought. The claims shall be clear and concise.

5.1 Claim 1 refers to a peptide comprising both L and D amino acid residues, having a net charge which is greater than +1, having a sequence of amino acid such that the corresponding amino acid sequence comprising only L-amino acid residues is not found in nature and cyclic derivatives thereof and said peptide shows selective cytolytic activity. Claim 1 refer to a mixture of 2 or more cytolytic peptides, to a complex of 2 or more cytolytic peptides said peptides being bundled by a covalent link and finally a random copolymer defined via different ratios of a hydrophobic, a positively and a D amino acid. Insofar as these peptides are defined solely by parameters and/ or functional features, it is at present impossible to determine what falls within the ambit of claim 1 (see also PCT Guidelines C-III, 4.7a). The same conclusion apply to claims 2 to 5.
Thus, at present claims 1 to 5 attempt to define the subject-matter in terms of the result to be achieved. The same objection of lack of clarity applies to claims 7 to 11, 15, 16 and 18.

It is reminded that claims which relate to a product solely identified by means of functional features and/or by parameters are claims for which no meaningful

opinion can usually be formed, because it cannot readily be identified what falls under the scope of protection.

5.2 The subject-matter of claim 19 is appended to claim 18 which is itself appended to an obscure claim 1. In any case the meaning of Lys/Leu 12 mers is unclear.

5.3 Claim 20 refers to a non-hemolytic cytolytic random copolymer which shows selective cytolytic activity and is consisting of different ratios of a hydrophobic, a positively charged and a D amino acid. Insofar as the product is defined solely by functional features and by parameters, it is open to interpretation what falls under the scope of said claim. It is reminded that the definition of a product via its parameters may be only allowed in very rare cases where the product claimed cannot be adequately defined in any other ways (see also PCT Guidelines C-III 4.7a). It is not the case for the claimed copolymer of claim 20. The same objection applies to claims 21.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

BEN-AMI, Paulina
Yeda Research & Development Co.Ltd.
Weizmann Institute of Science
P.O. Box 95
76100 Rehovot
ISRAEL

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	28.05.99

Applicant's or agent's file reference	IMPORTANT NOTIFICATION
9610 PCT 2	

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL98/00081	19/02/1998	20/02/1997

Applicant
YEDA RESEARCH AND DEVELOPMENT CO. LTD. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0 Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Zoglauer, H Tel.(+49-89) 2399-8718

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
9610 PCT 2		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL98/00081	19/02/1998	20/02/1997
International Patent Classification (IPC) or national classification and IPC C07K14/00		
Applicant YEDA RESEARCH AND DEVELOPMENT CO. LTD. et al.		

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   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

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3. This report contains indications relating to the following items:

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   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
14/09/1998	2.8. 05. 99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. (+49-89) 2399-0 Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer Pilat, D Telephone No. (+49-89) 2399 8668

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL98/00081

I. Basis of th report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-102 as originally filed

Claims, No.:

1-26 as originally filed

Drawings, sheets:

1/14-14/14 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5,6,12-17,20-26
	No:	Claims	1-4,7-11,18
Inventive step (IS)	Yes:	Claims	12-14,17
	No:	Claims	5,6,15,16,20-26
Industrial applicability (IA)	Yes:	Claims	1-26
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Ad Section I: Basis of the opinion

1) Reference is made to the following documents:

D1 Y Pouny & Y. Shai Biochemistry vol.31, n°39 6 October 1992 p.9482-90
D2 D. Rapaport et al. Biochemsitry vol.32, n°13 6 April 1993, p.3291-97
D3 Z. Oren & Y. Shai Biochemistry vol. 36, n°7 18 February 1997, p.1826-35
D4 Y. Shai & Z. Oren Journal of Biological Chemistry vol.271, n°13 29 March 1996, p.7305-8

Ad Section II :Priority

2) Priority (Article 8 PCT)

The priority document pertaining to the present application was not available at the time of establishing this IPER. Hence, it is based on the assumption that all claims enjoy priority rights from the filing date of the priority document. If it later turns out that this is not correct, the P documents cited in the international search report could become relevant to assess whether all claims satisfy the criteria set forth in Article 33(1) PCT.

Ad Section V :Reasoned statement under Rule 66.2(a)(ii); citations and explanations supporting such statement

3) Novelty (Article 33 (2) PCT)

3.1 D1 describes a set of Pardaxin analogues among which diastereoisomers (see table I). From this table only pardaxin D-$L^{18}L^{19}$-Par and D-P^{13}-Par were able to lyse the erythrocytes all the other were non-hemolytic (see p.9488, col.1 last full paragraph).
D2 describes pardaxin analogues in which amino groups were substituted by carboxylic groups (see table I).
D3 describes diastereoisomers of melittin (see table I). It states also that pardaxin and melittin have a net charge of +1 and +6 respectively (see p.1827, col.1 lines 12-20), that pardaxin is 40-100 fold less hemolytic than melittin towards human

erythrocytes and that they are both potent antibacterial peptides against Gram-positive and Gram-negative bacteria (see p.1827, col.1 lines 30-33). This document shows also that diastereoisomers peptides in the absence of an alpha helical structure caused abrogation of their cytotoxic effects on mammalian cells, although they retained antibacterial activity (see p.1827, col.1, lines 37-41 and p.1827, col.1 last paragraph and p.1832 col.2 lines 16-25).
Based on all these findings, D3 suggests designing antibacterial peptides, which lack pathological and pharmalogical effects induced by alpha-helical lytic cytolysin and proposed the synthesis of diastereoisomers of pardaxin which do not exert these activities. Furthermore, D-amino acid local substitution should enable a controlled clearance of the antibacterial peptides. Finally, D3 suggests that modulating both the hydrophobicity and net charge of diastereoisomers of linear cytotoxic polypeptides may be sufficient to design potent antibacterial polypeptides for the treatment of infectious diseases (see p.1834, col.1 last paragraph).
D4 discloses a set of pardaxin peptides, among which Tapar. This peptide has a net charge of +5 and a C-terminus converted to a positive one by transamination. Three diastereoisomers of Tapar have been synthesized (see table I and p.7306, col.1 "Results" first paragraph) and reduced the hemolytic properties of these analogues, whereas they all retained antibacterial activity (see p.7306, col.2 lines 24-27 and lines 32-35; p.7307 col.2 lines 5-18).

In view of the teaching of these documents, the subject-matter of claims 1 to 4 which comprises a non-hemolytic cytolytic agent having a selective cytolytic activity on pathogenic or malignant cells, is non-hemolytic or has a reduced hemolytic property, is a peptide comprising both L-amino acid residues and D-amino acid residues, has a net positive charge greater than +1 and has a sequence not found in nature is anticipated by the peptides n°6-13 in D1 table I; by peptide n°9 D2 table I (spontaneous succinylation is not found in nature); by the second peptide in D3 table I (transamination is very unlikely to occur in nature) and by the second to forth peptides in D4 table I (transamination is very unlikely to occur in nature). The same objection applies to a mixture of cytolytic peptides (see also D2, Fig.4). Thus claims 1 to 4 and 7 to 11 and 18 lack novelty.

3.2 None of the cited prior art discloses a cyclic pardaxin or a peptide as claimed in

claims 5, 6, 12-17 and 22-26. Thus, these claims seem to be novel.

4) Inventive step (Article 33 (3) PCT)

4.1 D2 describes the use of aminoethylamino groups (see D2, Table I peptide n°8 and 11). Furthermore, it is known that modification of the net charge may lead to a potent antibacterial polypeptides for the treatment of infectious diseases (D3 see p.1834, col.1 last paragraph). Therefore it appears trivial to add such an amino group to a selected peptide diastereoisomer in order to potentiate or lower its lytic efficiency. Thus, claim 5 lacks an inventive step.

4.2 Linear peptide of pardaxin are known (see point 3.1 above). Similarly the synthesis of cyclic peptides are also known in the art. Therefore to synthesize a cyclic form of pardaxin in order to provide an alternative to the linear peptide does not seem to be inventive unless the applicant shows a surprising technical effect associated with this particular form. At present claim 6 lacks an inventive step.

4.3 It is general knowledge that complex of peptides may be stabilized by a covalent linker molecule. In the light of the results obtained in D2 which shows that there is aggregation between peptides (Abstract, last sentence) the skilled person would have linked these diastereoisomers monomers together in order to stabilize them without any inventive activity. As a consequence claim 15 and 16 lack an inventive step.

4.4 The synthesis of a copolymer of a known peptide (see point 3.1 above) is an obvious alternative to the known monomers. The further selection of a copolymer having different ratios of hydrophobic, a positively charged and a D amino acid or a specific ratio seems to relate to an arbitrary choice the skilled person would have considered, since it was known that modulation of the hydrophobicity and net charge of diastereoisomers of linear cytotoxic polypeptides allows the design of potent antibacterial polypeptides (see D3 p.1834, col.1 last paragraph). Claims 20 and 21 are obvious.

4.5 Since the cytolytic peptides claimed in claim 1 are known and the application of these peptides for therapy, treatment of infectious diseases and against bacteria

are suggested (D3, p.1834, col.1, last paragraph) no inventive step can be seen for pharmaceutical composition containing such compounds. Therefore claims 22 to 26 are obvious.

4.6 The subject-matter of claim 12 which refer to a 6 mer, 8-mer, 12-mer peptide composed of leucine and lysine in which at least one third of the sequence is composed of D-amino acids seems to be new and is not suggested in the cited prior art when considered alone or in any combination. Consequently claim 12 as well as dependent claims 13 and 14 seems to involve an inventive step. For the same reasons claim 17 seems also to enjoy an inventive step.

Ad Section VIII : Certain observations on the international application.

5) Clarity (Article 6 PCT)

As defined in Article 6 PCT the claims shall **define** the matter for which protection is sought. The claims shall be clear and concise.

5.1 Claim 1 refers to a peptide comprising both L and D amino acid residues, having a net charge which is greater than +1, having a sequence of amino acid such that the corresponding amino acid sequence comprising only L-amino acid residues is not found in nature and cyclic derivatives thereof and said peptide shows selective cytolytic activity. Claim 1 refer to a mixture of 2 or more cytolytic peptides, to a complex of 2 or more cytolytic peptides said peptides being bundled by a covalent link and finally a random copolymer defined via different ratios of a hydrophobic, a positively and a D amino acid. Insofar as these peptides are defined solely by parameters and/ or functional features, it is at present impossible to determine what falls within the ambit of claim 1 (see also PCT Guidelines C-III, 4.7a). The same conclusion apply to claims 2 to 5.
Thus, at present claims 1 to 5 attempt to define the subject-matter in terms of the result to be achieved. The same objection of lack of clarity applies to claims 7 to 11, 15, 16 and 18.

It is reminded that claims which relate to a product solely identified by means of functional features and/or by parameters are claims for which no meaningful

opinion can usually be formed, because it cannot readily be identified what falls under the scope of protection.

5.2 The subject-matter of claim 19 is appended to claim 18 which is itself appended to an obscure claim 1. In any case the meaning of Lys/Leu 12 mers is unclear.

5.3 Claim 20 refers to a non-hemolytic cytolytic random copolymer which shows selective cytolytic activity and is consisting of different ratios of a hydrophobic, a positively charged and a D amino acid. Insofar as the product is defined solely by functional features and by parameters, it is open to interpretation what falls under the scope of said claim. It is reminded that the definition of a product via its parameters may be only allowed in very rare cases where the product claimed cannot be adequately defined in any other ways (see also PCT Guidelines C-III 4.7a). It is not the case for the claimed copolymer of claim 20. The same objection applies to claims 21.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 30 September 1998 (30.09.98)	
International application No. PCT/IL98/00081	**Applicant's or agent's file reference** 9610 PCT 2
International filing date (day/month/year) 19 February 1998 (19.02.98)	**Priority date** (day/month/year) 20 February 1997 (20.02.97)
Applicant SHAI, Yechiel et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

14 September 1998 (14.09.98)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Marie-José Devillard
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